ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF GABAPENTIN IN HUMAN PLASMA AND PHARMACEUTICAL DOSAGE FORMS

A

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Guided By:

Dr. S. A. Patel
M. Pharm., Ph. D.

Submitted By:

DUSHYANT PATEL
B. Pharm.

S. K. PATEL COLLEGE OF PHARMACEUTICAL EDUCATION AND RESEARCH

GANPAT UNIVERSITY, KHERVA - 382711

DIST. – MEHSANA, GUJARAT, INDIA.

CERTIFICATE

This is to certify that the research work embodied in this thesis entitled "*ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF GABAPENTIN IN HUMAN PLASMA AND PHARMACEUTICAL DOSAGE FORMS*" was carried out by **Mr. Dushyant B. Patel** under my guidance. The work is original, bonafide and it is up to my satisfaction.

Guided by:

Dr. S. A. Patel
M.Pharm., Ph. D. (Asst. Professor)
Dept. of Pharm. Quality Assurance,
Shree S. K. Patel College of
Pharmaceutical Edu. & Res.
Kherva, Mehsana

Head of the Department:

Dr. S.S.Pancholi
M.Pharm., Ph. D. (Professor)
Dept. of Pharm. Quality Assurance,
Shree S. K. Patel College of
Pharmaceutical Edu. & Res.
Kherva, Mehsana

Principal:

Dr. N.J.Patel

M.Pharm, Ph. D.
Shree S. K. Patel College of
Pharmaceutical Edu. & Res.
Kherva, Mehsana

Date:

Place:

DECLARATION

I hereby declare that this thesis entitled ***"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF GABAPENTIN IN HUMAN PLASMA AND PHARMACEUTICAL DOSAGE FORMS"*** **is** based on original work carried out by me in the laboratories of Torrent Research Centre, Gandhinagar and Shree S.K.Patel College of Pharmaceutical Education And Research, Kherva. This work has not been submitted earlier to any other University or College.

Date: **(Dushyant B. Patel)**

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List of Abbreviations

Abbreviations	Description
arb	Arbitrary Unit
ACN	Acetonitrile
β	Beta
BA	Bioanalytical
BCG	Bromocresol Green
BLQ	Below Limit of Quantification
BTB	Bromothymol blue
C_{max}	Concentration Maximum
cc	Cubic centimeter
CDC	Centers for Disease Control and Prevention
Conc.	Concentration
CS	Calibration Standard
CV	Coefficient of Variance
FDA	Food and Drug Administration
GBP	Gabapentin
HPLC	High Performance Liquid chromatography
hrs	Hours
HQC	High Quality Control
ICH	International Conference on Harmonization
i.d.	Internal Diameter
IS	Internal Standard
LC-MS/MS	Liquid Chromatography-Mass Spectrometry- Mass Spectrometry
LEV	Levetiracetam
LLOQ	Lower Limit of Quantification
LQC	Low Quality Control
min	Minutes
ml	Milliliter
mm	Millimeter
MP	Mobile phase

mTorr	Milli Torr
MS	Mass Spectrometry
MQC	Medium Quality Control
NA	Not Available
ng	Nanogram
NIST	National Institute of Standards and Technology
No.	Number
OPA	O-phosphoric acid
QC	Quality Control
r	Correlation Coefficient
RPM	Revolution per minute
RT	Retention Time
SD	Standard Deviation
sec	Seconds
SOP	Standard Operating Procedure
SOTP	Standard Operating Test Procedure
temp.	Temperature
ULOQ	Upper Limit of Quantification
V	Volts
VGB	Vigabatrin
v/v	Volume/Volume
v/v/v	Volume/Volume/Volume
Vol	Volunteer
µg	Microgram
µl	Microliter
%	Percentage
mM	Milli molar
STD	Standard
SYS SUIT	System Suitability

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION TO EPILEPSY:

- Epilepsy is a common chronic neurological disorder that is characterized by recurrent unprovoked seizures[1, 2]. These seizures are transient signs and/or symptoms due to abnormal, excessive or synchronous neuronal activity in the brain[3]. About 50 million people worldwide have epilepsy at any one time[4]. Epilepsy is usually controlled, but not cured, with medication, although surgery may be considered in difficult cases. However, over 30% of people with epilepsy do not have seizure control even with the best available medications [5]. Not all epilepsy syndromes are lifelong – some forms are confined to particular stages of childhood. Epilepsy should not be understood as a single disorder, but rather as a group of syndromes with vastly divergent symptoms but all involving episodic abnormal electrical activity in the brain.

1.1.1 CLASSIFICATION:

Epilepsies are classified in six ways:

1. By their first cause (or etiology).
2. By the observable manifestations of the seizures, known as semiology.
3. By the location in the brain where the seizures originate.
4. As a part of discrete, identifiable medical syndromes.
5. By the event that triggers the seizures, as in primary reading epilepsy
6. Musicogenic epilepsy.

1.1.2 DIAGNOSIS:

- The diagnosis of epilepsy requires the presence of recurrent, unprovoked seizures; accordingly, it is usually made based on the medical history. Imaging and measurement technologies such as electroencephalography (EEG), magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), positron emission tomography (PET), and magneto encephalography (MEG) may be useful to discover an etiology for the epilepsy, discover the affected brain region, or classify the epileptic syndrome, but these studies are not useful in making the initial diagnosis.
- Long-term video-EEG monitoring for epilepsy is the gold standard for diagnosis, but it is not routinely employed owing to its high cost, low availability and inconvenience. Convulsive or other seizure-like activity, non-epileptic in origin, can be observed many

other medical conditions. These non-epileptic seizures can be hard to differentiate in and may lead to misdiagnosis.

- Epilepsy covers conditions with different etiologies, natural histories and prognoses, each requiring different management strategies. A full medical diagnosis requires a definite categorization of seizure and syndrome types[6].
- Many people are misdiagnosed, because doctors unfamiliar with the symptoms believe that their patients have another illness, because they are not adequately trained to recognize the early symptoms including odd tastes or smells. Approximately 80% have petit mal seizures which are harder to spot.
- According to the National Institute of Neurological Disorders and Stroke, if a person has had two or more seizures they are considered to have epilepsy. There are however exceptions: seizures caused by fever (febrile seizures), those not due to abnormal electrical activity in the brain (no epileptic events), and seizures that occur during pregnancy (eclampsia) are not counted.

1.1.3 PRECIPITANTS:

- The diagnosis of epilepsy usually requires that the seizures occur spontaneously. Nevertheless, certain epilepsy syndromes require particular precipitants or triggers for seizures to occur. These are termed reflex epilepsy. For example, patients with primary reading epilepsy have seizures triggered by reading. Photosensitive epilepsy can be limited to seizures triggered by flashing lights. Other precipitants can trigger an epileptic seizure in patients who otherwise would be susceptible to spontaneous seizures. For example, children with childhood absence epilepsy may be susceptible to hyperventilation. In fact, flashing lights and hyperventilation are activating procedures used in clinical EEG to help trigger seizures to aid diagnosis.
- Finally, other precipitants can facilitate, rather than obligatory trigger, seizures in susceptible individuals. Emotional stress, sleep deprivation, sleep itself, and febrile illness are examples of precipitants cited by patients with epilepsy. Notably, the influence of various precipitants varies with the epilepsy syndrome [7].

1.1.4 SEIZURE TYPES:

- Seizure types are organized firstly according to whether the source of the seizure within the brain is localized (partial or focal onset seizures) or distributed (generalized seizures). Partial seizures are further divided on the extent to which consciousness is affected. If it is unaffected, then it is a simple partial seizure; otherwise it is a complex partial (psychomotor) seizure. A partial seizure may spread within the brain - a process known

as secondary generalization. Generalized seizures are divided according to the effect on the body but all involve loss of consciousness. These include absence (petit mal), myoclonic, clonic, tonic, tonic-clonic (grand mal) and atonic seizures.

1.1.5 TREATMENT [8-14]:

- Epilepsy is usually treated with medication prescribed by a physician; primary caregivers, neurologists, and neurosurgeons all frequently care for people with epilepsy. In some cases the implantation of a stimulator of the vagus nerve, or a special diet can be helpful. Neurosurgical operations for epilepsy can be palliative, reducing the frequency or severity of seizures; or, in some patients, an operation can be curative.

1.1.5.1 Medication for Epilepsy:

1. Drugs acting through Na^+ channels

Examples:

- Phenytoin
- Diphenylhydantoin (DPH)
- Phenobarbitone
- Trimethadione
- Carbamazepine
- Lamotrigine

2. Drugs acting through GABA

Examples:

- Sodium valproate
- Diazepan and Clonazepam
- Midazolam
- Gabapentin
- Vigabatrin
- Clobazam

3. Drugs acting through NMDA receptor inhibition

Examples:

- Flunarizine
- Zonisamide
- Topiramate

4. Non pharmacological therapy

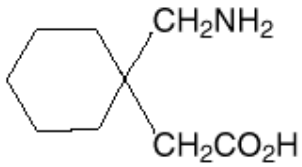
Examples:

- Vagus nerve stimulation
- Ketogenic diet

- Surgery

1.2 DRUG PROFILE[15-17]:

- **Drug name:** Gabapentin
- **Proprietary name:**
  - Neurontin , Aclonium
- **Category:** Antiepileptic drug
- **Iupac name:**
  - 2-[1-(aminomethyl)cyclohexyl]acetic acid
- **Structure:**



1.2.1 PHYSICOCHEMICAL PROPERTIES:

- **Molecular Formula:** $C_9H_{17}NO_2$
- **Molecular Weight:** 171.24 gm/mol
- **Melting Point:** 162-166° C
- **pKa:** 3.68, 10.70
- **Isoelectric Point:** 7.14
- **Solubility:**
  - Ethanol : 1 in 90
  - Water : 1 in 10
- **Partition Coefficient:**
  - Octanol/Water: 0.075
- **Log P:** -1.10
- It is white to off-white crystalline solid

1.2.2 PHARMACOKINETICS PROPERTIES:

- Oral Absorption : ~60%(Dose Dependent)
- Presystemic Metabolism : NIL
- Plasma Half Life : 5-7 hrs
- Volume Of Distribution : 0.6 to 0.8 l/kg
- Plasma Protein Binding : < 3%
- Dose(mg) : 300 mg 600 mg 900 mg
- Cmax(mg/l) : 2.7 mg/l 3.29 mg/l 4.02 mg/l

1.2.3 MECHANISM OF ACTION:

- The mechanism by which GBP exerts its analgesic action is unknown, but in animal models of analgesia, GBP prevents allodynia (pain-related behavior in response to a normally innocuous stimulus) and hyperalgesia (exaggerated response to painful stimuli). In particular, GBP prevents pain-related responses in several models of neuropathic pain in rats or mice (e.g. spinal nerve ligation models, streptozocin-induced diabetes model, spinal cord injury model, acute herpes zoster infection model). GBP also decreases pain-related responses after peripheral inflammation (carrageenan footpad test, late phase of formalin test). GBP did not alter immediate pain-related behaviors (rat tail flick test, formalin footpad acute phase, acetic acid abdominal constriction test, footpad heat irradiation test). The relevance of these models to human pain is not known.
- The mechanism by which GBP exerts its anticonvulsant action is unknown, but in animal test systems designed to detect anticonvulsant activity, GBP prevents seizures as do other marketed anticonvulsants. GBP exhibits antiseizure activity in mice and rats in both the maximal electroshock and pentylenetetrazole seizure models and other preclinical models (e.g., strains with genetic epilepsy, etc.). The relevance of these models to human epilepsy is not known.
- GBP is structurally related to the neurotransmitter GABA (gamma-aminobutyric acid) but it does not modify $GABA_A$ or $GABA_B$ radioligand binding, it is not converted metabolically into GABA or a GABA agonist, and it is not an inhibitor of GABA uptake or degradation. GBP was tested in radioligand binding assays at concentrations up to 100 μM and did not exhibit affinity for a number of other common receptor sites, including benzodiazepine, glutamate, N-methyl-D-aspartate (NMDA), quisqualate, kainate, strychnine-insensitive or strychnine-sensitive glycine, alpha 1, alpha 2, or beta adrenergic, adenosine A1 or A2, cholinergic muscarinic or nicotinic, dopamine D1 or D2, histamine H1, serotonin S1 or S2, opiate mu, delta or kappa, cannabinoid 1, voltage-sensitive calcium channel sites labeled with nitrendipine or diltiazem, or at voltage-sensitive sodium channel sites labeled with batrachotoxinin A 20-alpha-benzoate. Furthermore, GBP did not alter the cellular uptake of dopamine, noradrenaline, or serotonin.
- *In vitro* studies with radiolabeled GBP have revealed a GBP binding site in areas of rat brain including neocortex and hippocampus. A high-affinity binding protein in animal brain tissue has been identified as an auxiliary subunit of voltage-activated calcium channels. However, functional correlates of GBP binding, if any, remain to be elucidated.

1.2.4 RECOMMENDED DOSAGE:

- People over the age of 12 should be started on 300 mg GBP taken three times a day. The dose can be increased up to a total of 1,800 mg per day. In some instances, doses of up to 3,600 mg per day have been tolerated.
- Children should receive a dosage of 10–15 mg per kg of body weight per day, divided into three equal doses.
- Chronic pain may be treated with 300–3,600 mg per day, divided into three equal doses.
- When GBP is used for bipolar disorder, the starting dose is usually 300 mg taken at bedtime. Depending on the patient's response, the dose can be increased every four to seven days. Many people receive maximum therapeutic benefit at 600 mg per day, although some people have required up to 4,800 mg per day.

1.2.5 SIDE EFFECTS:

- Patients who experience the following side effects of GBP should check with their doctor immediately. These include more common side effects, such as unsteadiness, clumsiness, and uncontrollable back-and-forth eye movements or eye rolling. Less common side effects include depression, irritability, other mood changes or changes in thinking, and decreased memory. Rare side effects include pain in the lower back or side, difficulty urinating, fever and/or chills, cough, or hoarseness.
- Children under age 12 who have the following more common side effects should also check with their doctor immediately: aggressive behavior, irritability, anxiety, difficulty concentrating and paying attention, crying, depression, mood swings, increased emotionality, hyperactivity, suspiciousness or distrust.
- Multiple side effects often occur when a patient starts taking GBP. While these side effects usually go away on their own, if they last or are particularly troublesome, the patient should consult a doctor. More common side effects that occur when first starting to take GBP include blurred or double vision, muscle weakness or pain, swollen hand, feet, or legs, trembling or shaking, and increased fatigue or weakness. Less common side effects that occur when initiating GBP treatment include back pain, constipation, decreased sexual drive, diarrhea, dry mouth and eyes, frequent urination, headache, indigestion, low blood pressure, nausea, ringing in the ears, runny nose, slurred speech, difficulty thinking and sleeping, weight gain, twitching, nausea and/or vomiting, weakness.

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CHAPTER 2: REVIEW OF LITERATURE

2.1 REVIEW OF LITERATURE:

TABLE NO: 2.1 OFFICIAL ANALYTICAL METHOD

SR NO	TITLE	METHOD	EXPERIMENTAL CONDITION
1.	USP official Method [1]	HPLC	**Column:** 4.6 mm×25 cm with L1 packing **MP:** ACN: phosphate buffer (24:76, v/v) (pH:1.8) **Detection method:** UV detection (215 nm) **Flow rate:** 1ml/min

TABLE NO: 2.2 REPORTED ANALYTICAL METHODS

SR NO	TITLE	METHOD	EXPERIMENTAL CONDITION
1.	Rapid quantification of gabapentin in human plasma by LC- MS/MS[2].	LC-MS/MS	**Column:** Waters symmetry C18 RP **MP:** ACN: 10 mM Amm. formate (80:20, v/v, pH:3.0) **IS:** 1,1-Cyclohexane diacetic acid monoamide **MS mode:** MRM
2.	Determination of gabapentin in human plasma using hydrophilic interaction LC- MS/MS[3].	LC-MS/MS	**Column:** Atlantis HILIC silica **MP:** ACN: 100 mM Amm. formate (85:15, v/v) (pH:3.0) **IS:** Metformin **MS mode:** MRM
3.	Validated LC-MS/MS method for quantification of gabapentin in human plasma: application to pharmacokinetic and BE studies in Korean volunteers[4].	LC-MS/MS	**Column:** Gemini C18 **MP:** ACN: 10 mM Amm. acetate (20:80, v/v, pH:3.2) **IS:** (S)-(+)-alpha-amino cyclohexanepropionic acid hydrate

4.	Gabapentin quantification in human plasma by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry. Application to bioequivalence study[5].	LC-MS/MS	**Extraction method:** Protein precipitation **Column:** C4 RP **MS mode:** MRM
5.	Quantitative determination of vigabatrin and gabapentin in human serum by gas chromatography - mass spectrometry[6].	GC-MS	**Column:** polydimethylsiloxane **IS**: Cyclobarbital **MS mode**: SIM
6.	Analysis of gabapentin in serum and plasma by solid-phase extraction and GC-MS for therapeutic drug monitoring[7].	GC-MS	**Extraction method:** SPE **Derivatization reagent :** MTBSTFA
7.	Determination of gabapentin in pharmaceutical preparations by HPTLC[8].	HPTLC	**Plate:** Aluminum-backed silica gel 60 F^{254} HPTLC plates **MP:** n-butanol: water :glacial acetic acid (3:3:2, v/v) **IS:** Histamine hydrochloride
8.	Development and application of a validated HPLC method for the determination of gabapentin and its major degradation impurity in drug products[9].	HPLC	**Column:** Brownlee spheri-5 cyano **MP:** ACN:10 mM KH_2PO_4/10 mM K_2HPO_4 (pH 6.2,8:92 v/v) **Detection method:** UV detection at 210 nm
9.	A HPLC micro method for the simultaneous determination of vigabatrin and gabapentin in serum[10].	HPLC	**Column:**125 x 3-mm ID Hypersil BDS C-18 column, 3-µ mini-bore **MP :** phosphate buffer (pH 6.5):ACN:methanol:water

10.	Automated microanalysis of gabapentin in human serum by high-performance liquid chromatography with fluorometric detection[11].	HPLC	**Extraction method:** PP **Derivatization reagent**: o-phthaldialdehyde reagent **Column:** Waters 5 µ RP column (10 cmx4.6 mm) **IS:** 1-(aminomethyl) cycloheptane acetic acid **MP:** 0.02 M phosphate buffer (pH 4.5):ACN (50:50, v/v) **Detection :** Fluorimetry (EX: 230 nm, EM: 420 nm)
11.	Determination of gabapentin in plasma by high-performance liquid chromatography[12].	HPLC	**Derivatization reagent:** o-phthalaldehyde-3-mercaptopropionic acid **IS:** [1-(aminomethyl)-cycloheptaneacetic acid **Column:** Beckman Ultrasphere 5 microns RP **MP:** 0.33 M acetate buffer (pH 3.7): methanol : ACN (40:30:30, v/v/v) **Detection method :** Fluorimetry (EX: 330 nm, EM: 440 nm)
12.	Optimization of an HPLC method for determination of gabapentin in dosage forms through derivatization with 1-fluoro-2, 4-dinitrobenzene[13].	HPLC	**IS:** Amlodipine **Derivatization reagent :** 1-fluoro-2,4-dinitrobenzene **Column :** Nova-Pak C(18) **MP:** ACN: sodium dihydrogenphosphate (pH 2.5; 0.05 M) (70:30, v/v) **Detection:** UV(360 nm)

13.	Determination of gabapentin in human plasma and urine by high-performance liquid chromatography with UV-vis detection[14].	HPLC	**Extraction method:** SPE C18 cartridge **Derivatization reagent:** 1,2-naphthoquinone-4-sulphonic acid sodium salt (NQS) **Column:** C18 **MP:** ACN:10mM OPA (pH 2.5,v/v) **IS:** Baclofen
14.	Sensitive high-performance liquid chromatographic quantitation of gabapentin in human serum using liquid-liquid extraction and pre-column derivatization with 9-fluorenylmethyl chloroformate[15].	HPLC	**Extraction method:** LLE **Extracting solvent:** dichloromethane : 2 propanol (1:1, v/v) **Derivatization reagent**: 9-fluorenyl methyl chloroformate (FMOC-Cl) **Column:** Shimpack CLC-C18 (150 mm x 4.6 mm) **MP:** methanol:0.05 M sodium phosphate buffer (73:27, v/v; pH of 3.9) containing 1 ml/l triethylamine
15.	Simple and sensitive liquid chromatographic method with fluorimetric detection for the analysis of gabapentin in human plasma[16].	HPLC	**Derivatization reagent:** (2-naphthoxy)acetyl chloride (NAC) **Column:** phenyl-hexyl column **MP:** sodium acetate buffer (100 mM; pH 5.0):methanol (32:68, v/v) **Detection method:** Fluorimetry (EX: 225 nm, EM: 360 nm)

16.	Simultaneous isocratic HPLC determination of vigabatrin and gabapentin in human plasma by dansyl derivatization[17].	HPLC	**Derivatization reagent:** Dansyl chloride **Column:** Micro bondapak C-18, 10 µ , 300 x 3.9 mm **MP:** 50 mmol/l NaH_2PO_4 in 40% ACN **Detection method:** Fluorimetry (EX: 318 nm, EM: 510 nm)
17.	Determination of gabapentin in serum using solid-phase extraction and gas-liquid chromatography[18].	GLC	**Extraction method:** SPE on C18 solid-phase column **Derivatization reagent:** MTBSTFA plus 1% tBDMCS **Column :** HP-1 **Detector:** FID **IS:** [(1-aminomethyl-1-cycloheptyl) acetic acid]
18.	Determination of gabapentin in serum by capillary electrophoresis[19].	CE	**Derivatization reagent:** fluorescamine **Detection method:** UV-fluorescence detection
19.	Determination of gabapentin in human plasma by capillary electrophoresis with laser-induced fluorescence detection and acetonitrile stacking technique[20].	CE	**Derivatization reagent:** 6-Carboxyfluorescein succinimidyl ester (CFSE) **Buffer:** 50 mM sodium borate (pH 9.5) **Detection method:** Fluorimetry (EX: 488 nm, EM: 520 nm)
20.	Spectrofluorimetric determination of vigabatrin and gabapentin in urine and dosage forms through derivatization with fluorescamine[21].	Spectrofluorimetry	**Derivatization reagent:** fluorescamine **Detection method:** Spectrofluorimetry (EX: 390 nm, EM: 472 nm)

21.	Spectrofluorimetric determination of vigabatrin and gabapentin in dosage forms and spiked plasma samples through derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole[22].	Spectrofluorimetry	**Derivatization reagent:** 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole **Detection method:** Fluorimetry (EX: 465 nm, EM: 532 nm)
22.	Colorimetric determination of gabapentin in pharmaceutical formulation[23].	Colorimetry	Based on following reaction 1) GBP with vanillin (Duquenois reagent) in the presence of McIlvain buffer pH 7.5 and the color developed was measured at 376 nm. 2) The primary amino group of GBP with ninhydrin reagent in N, N'-dimethylformamide (DMF) medium producing colored product which absorbs maximally at 569 nm. 3) The reaction of gabapentin with p-benzoquinone (PBQ) to form a colored product with λmax at 369 nm.
23.	Determination of the antiepileptics vigabatrin and gabapentin in dosage forms and biological fluids using Hantzsch reaction[24].	Colorimetry	**Reaction:** condensation of the drugs through their amino groups with acetylacetone and formaldehyde according to Hantzsch reaction yielding the highly fluorescent dihydro-pyridine derivatives. **Color developed:** yellowish orange measured at 410 nm &

			415 nm for VGB and GBP, respectively.
24.	Rapid chemiluminometric determination of gabapentin in pharmaceutical formulations exploiting pulsed-flow analysis[25].	Chemiluminometry	**Reaction:** The luminol-hypochlorite reaction in the presence of GBP.

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CHAPTER 3: AIM OF THE PRESENT WORK

3.1 AIM OF THE PRESENT WORK:

- Nowadays it is important to estimate drug concentration in biological fluid to achieve better therapeutic effect and to check out content of drug in pharmaceutical dosage forms.
- Many methods are available to estimate GBP in human plasma, but our aim behind this project is to develop accurate, precise and validated method for estimation of GBP which has higher selectivity and specificity, accuracy and precision which can give reliable results without interference from biological matrix.
- Also no single extractive spectrophotometric method is available to estimate GBP in pharmaceutical dosage forms.

The specific aim of the research work was

- To develop and validate LC-MS/MS method for estimation of GBP in human plasma.
- To develop and validate spectrophotometric method for determination of GBP in pharmaceutical dosage forms.

CHAPTER: 4 DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR ESTIMATION OF GABAPENTIN IN HUMAN PLASMA

4.1 INTRODUCTION TO LC-MS/MS:

4.1.1 MASS SPECTROMETRY (MS):

- Mass spectrometry (MS) has an important place amongst the various spectrometric techniques for molecular analysis. The phenomenon of deflection of ions in electric or magnetic field first was proposed by Wien in 1898. A mass spectrometer for general use however available after 1930 only. At present it is most sensitive method of molecular analysis. Moreover, it has the potential to yield information on the relative molecular mass and the structure of the analyte. The principle of MS is production of ions that are subsequently separated or filtered according to their mass-to-charge (m/z) ratio and detected. The resulting mass spectrum is a plot of the (relative) abundance of the generated ions as a function of m/z ratio[1]. Thus MS is a very sensitive, highly selective and quantitative analytical technique. Sample size is usually in microgram to the nanogram range and fragmentation patterns are highly reproducible even for multi-component mixtures.
- The Mass spectrometer nowadays is a highly sophisticated and computerized instrument[1-4]. It basically consists of five parts: sample introduction, ionization, mass analysis, ion detection and data handling. Sample introduction systems comprise controlled leaks, through which a sample vapor is introduced from a reservoir, various direct insertion probes for the introduction of solids and low-volatility liquid and combination with various chromatographic techniques. The ionization of the analytes can be performed in a number of ways the available ionization techniques can be classified in four groups, i.e. electron ionization, chemical ionization, desorption ionization and nebulization ionization[1,5]. Proton transfer, charge exchange, electrophilic addition and anion abstraction reaction used to produce positively charged ions , M^+ or MH^+. Negative ions are produce by proton transfer or abstraction and anion attachment. After the production of ions, these are analyzed according to their m/z ratio in time or space with the mass analyzer. This means that a singly charged molecule with molecular mass of 400 will give a peak at m/z 400, while a molecule carrying 40 charges and a molecular mass of 16,000 will also give a

peak at 400 m/z. Five types of mass analyzers are currently available, i.e., magnetic sector [6], quadrupole mass filter [6], quadrapole ion trap [6,7], time-of-flight[6,8] and fourier-transform ion-cyclotron resonance instruments[6,9]. There is not one ideal mass analyzer. The choice depends on the application.

- In practice, most interfaces for coupling of LC with MS have been developed on quadrupole instrument. The detection of ions after the mass analysis is mostly performed by means of an electron multiplier. The signal of the electron multiplier is fed to multichannel analyzer to perform ion counting. In most modern instruments a high-voltage conversion dynode is used in both positive-ion and negative-ion detection**.** A mass spectrometer produces an enormous amount of data. The most important type of output are mass spectrum, mass chromatogram and total ion chromatogram. The mass spectrum is a plot of relative intensity as a function of the m/z. It contains a base peak, which is the peak with highest intensity used to normalize the mass spectrum. The mass spectrum is also contains fragment peaks, isotope peak and background peak.
- It is an important spectrometric technique for molecular mass analysis. At present MS is the most sensitive method for molecular analysis. Mass spectrometry could be considered as an analytical technique that involves the study in the gas phase of ionized molecules with the aim of one or more of the following:
    - Molecular weight determination.
    - Structural characterization.
    - Gas phase reactivity study.
    - Qualitative and quantitative analysis of components in a mixture.
- In analysis by LC/MS, a sample is injected onto an LC column. The sample is then separated into its various components. The components elute from the LC column and pass into the mass spectrometer where they are analyzed. Analysis by direct infusion or flow injection provides no chromatographic separation of components in the sample before it passes into the mass spectrometer. The data from the mass spectrometer are then stored and processed by the data system. In LC-MS sample is introduce by three ways. They are as -
    - Using the syringe pump (Direct infusion).
    - Using the divert/inject valve fitted with a sample loop and LC (flow injection analysis).
    - Using the divert/inject valve and HPLC fitted with column (LC-MS).

Ionization sources:

- In LC-MS various types of ionization sources are use. They are as-

(A) For small volatile molecules

- Electron Ionization (EI).
- Chemical Ionization (CI).

(B) For small or non-volatile molecules

- Electrospray ionization (ESI).
- Matrix-assisted Laser Desorption Ionization (MALDI)
- Field desorption (FD).
- Fast Atom Bombardment (FAB).

Instrumentation:

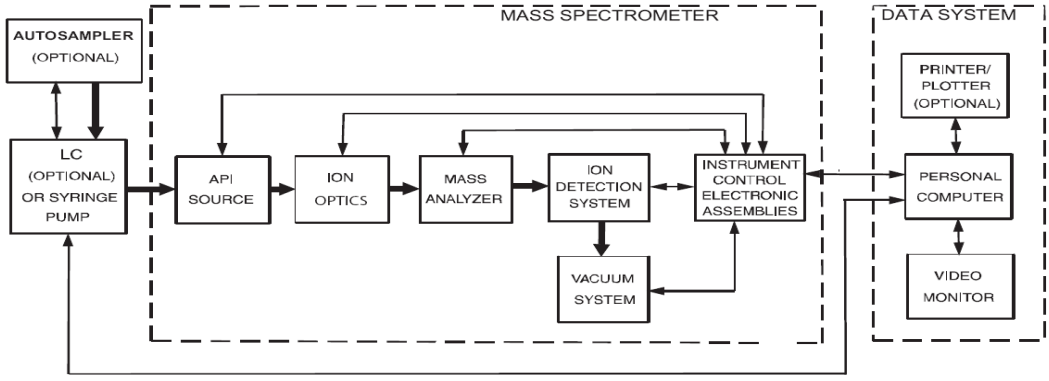


Figure 4.1 Schematic diagram of Mass spectroscopy

- The mass spectrometer consists of an ionization source, ion guides, triple-stage mass analyzer, and ion detection system. The ion guides, mass analyzer, ion detection system, and part of the ionization source are enclosed in a vacuum manifold. Ionization of the sample takes place in the ionization source. The specific process used to ionize the sample is referred to as the ionization mode. The ions produced in the ionization source are transmitted by the ion guides into the mass analyzer, where they are separated according to their mass-to-charge ratio. The polarity of the potentials applied to the lenses in the ionization source and ion guides determines whether positively charged ions or negatively charged ions are transmitted to the mass analyzer. It can analyze positively or negatively charged ions (called the positive or negative ion polarity mode).
- The ions produced in the ionization source are filtered according to their mass-to charge ratios (mass analyzed) by the triple-stage mass analyzer, which can perform either one or two stages of mass analysis. When the system is operated as a conventional mass spectrometer with one stage of mass analysis, the sample is ionized in the ion source and the ion products are subjected to mass analysis in the first rod assembly. The resulting

mass-selected ions are then transmitted (through the second and third rod assemblies) to the ion detection system.

- When the system is operated as a tandem mass spectrometer, as before, the sample is ionized in the ion source and is mass analyzed by the first rod assembly. In this case, however, mass-selected ions exiting the first rod assembly are allowed to collide with an inert gas in the second rod assembly and fragment to produce a new set of ion products. (The second rod assembly is surrounded by a chamber called the collision cell. The collision cell can be pressurized with an inert gas.) The secondary ion products then undergo a further mass analysis in the third rod assembly and selected ions are detected. In this mode, two stages of analysis are performed. In the first stage, ion production occurs in the ion source, and the first mass analyzer carries out mass analysis. In the second stage of analysis, formation of new ionic species occurs in the collision cell, and the second mass analyzer performs mass analysis. Two stages of mass analysis yield far greater chemical specificity than a single stage can achieve, because two discrete but directly related sets of masses can be selected and determined.
- Systems can be used to elucidate the structures of pure organic compounds and the structures of the components within mixtures. Each ionic fragment of a molecule formed in the ion source and separated in a first stage of mass analysis can be further fragmented and then further separated in a second stage of mass analysis to build up an entire structure for the molecule, piece by piece. Thus, all pathways for the formation and fragmentation of each ion in the mass spectrum can be investigated.
- The two stages of mass analysis, with resultant reduction of chemical noise in the final mass spectrum, make analysis very selective and sensitive.
- Each sequence of single or triple-stage mass analysis of the ions is called a scan. The system uses several different scan modes and different scan types to filter, fragment, or transmit ions in the mass analyzer. The ability to vary the scan mode and scan type, as well as the ionization and ion polarity modes, affords the user great flexibility in the instrumentation for solving complex analytical problems.

Ion polarity modes:

- Two ion polarity modes: Positive or negative.
- Both positively charged and negatively charged ions are formed in the ionization source of the mass spectrometer. The mass spectrometer can control whether positive ions or negative ions are transmitted to the mass analyzer for mass analysis by changing the polarity of the potentials applied to the ionization source and ion guides. The ion guides are located between the ionization source and the mass analyzer.

- The information obtained from a positive-ion mass spectrum is different from and complementary to that obtained from a negative-ion spectrum.
- Thus, the ability to obtain both positive-ion and negative-ion mass spectra aids you in the qualitative analysis of your sample. You can choose the ion polarity mode and ionization mode to obtain maximum sensitivity for the particular analysis of interest.

Ionization modes:

- Two ionization modes:
    - Electrospray ionization (ESI)
    - Atmospheric pressure chemical ionization (APCI)

Electrospray ionization:

- The electrospray ionization (ESI) mode transforms ions in solution into ions in the gas phase. Many samples that previously were not suitable for mass analysis (for example, heat-labile compounds or high molecular weight compounds) can be analyzed by the use of ESI. ESI can be used to analyze any polar compound that makes a preformed ion in solution.
- With ESI, the range of molecular weights that can be analyzed by the mass spectrometer is greater than 100,000 U, due to multiple charging. ESI is especially useful for the mass analysis of polar compounds, which include biological polymers (for example, proteins, peptides, glycoproteins, and nucleotides), pharmaceuticals & their metabolites and industrial polymers (for example, polyethylene glycols).

In ESI, ions are produced and analyzed as follows:

- The sample solution enters the ESI needle, to which a high voltage is applied.
- The ESI needle sprays the sample solution into a fine mist of droplets that are electrically charged at their surface.
- The electrical charge density at the surface of the droplets increases as solvent evaporates from the droplets.
- The electrical charge density at the surface of the droplets increases to a critical point known as the Rayleigh stability limit. At this critical point, the droplets divide into smaller droplets because the electrostatic repulsion is greater than the surface tension. The process is repeated many times to form very small droplets.
- From the very small, highly charged droplets, sample ions are ejected into the gas phase by electrostatic repulsion.
- The sample ions enter the mass spectrometer and are analyzed.

- The ESI mode use in either positive or negative ion polarity mode. The ion polarity mode of choice is determined by the polarity of the preformed ions in solution: Acidic

molecules form negative ions in solution, and basic molecules form positive ions. The ejection of sample ions from droplets is facilitated if the ionic charge and surface charge of the droplet are of the same polarity. Thus, a positively charged needle is used to analyze positive ions and a negatively charged needle is used to analyze negative ions.

- Sample ions can carry a single charge or multiple charges. The number of charges carried by the sample ion depends on the structure of the analyte of interest and the carrier solvent. The ESI process is affected by droplet size, surface charge, liquid surface tension, solvent volatility, and ion solvation strength. Large droplets with high surface tension, low volatility, strong ion solvation, low surface charge, and high conductivity prevent good electrospray.

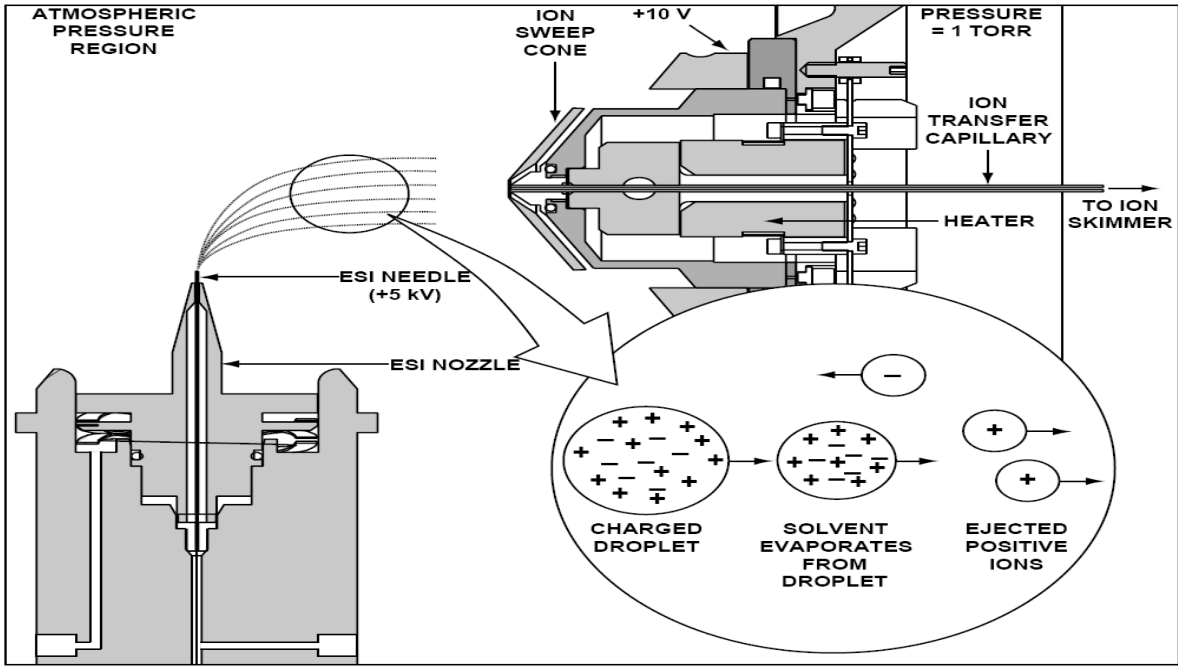


Figure 4.2 ESI process in the positive ion polarity mode

- Organic solvents such as methanol, acetonitrile, and isopropyl alcohol are superior to water for ESI. Volatile acids and bases are good, but salts above 10 mM concentration and strong acids and bases are extremely detrimental.

Atmospheric pressure chemical ionization:

- Atmospheric pressure chemical ionization (APCI) is a soft ionization technique, but not as soft as ESI. APCI is used to analyze compounds of medium polarity that have some volatility.
- In APCI, ions are produced and analyzed as follows:
    - The APCI nozzle sprays the sample solution into a fine mist of droplets.

- The droplets are vaporized in a high temperature tube (the vaporizer).
- A high voltage is applied to a needle located near the exit end of the tube. The high voltage creates a corona discharge that forms reagent ions through a series of chemical reactions with solvent molecules and nitrogen sheath gas.
- The reagent ions react with sample molecules to form sample ions.
- The sample ions enter the mass spectrometer and are analyzed.

- APCI is a gas phase ionization technique. Therefore, the gas phase acidities and basicities of the analyte and solvent vapor play an important role in the APCI process.
- In the positive-ion mode, sample ionization occurs in a series of reactions that start with the electron-initiated cation formation. Typical examples of primary, secondary, and adduct ion formation are shown below:

Primary ion formation

$$e^- + N_2 \rightarrow N_2^{+\cdot} + 2e-$$

Secondary ion formation

$$N_2^+ + H_2O \rightarrow N_2 + H_2O^{+\cdot}$$

$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + HO.$$

Proton transfer

$$H_3O^+ + M \rightarrow (M+H)^+ + H_2O$$

- In negative-ion mode, $(M-H)^-$ is typically formed by the abstraction of a proton by $OH-$.
- APCI is typically used to analyze small molecules with molecular weights up to about 1500 U. APCI is a very robust ionization technique. It is not affected by minor changes in most variables, such as changes in buffers or changes in buffer strength.
- APCI use in positive or negative ion polarity mode. For most molecules, the positive-ion mode produces a stronger ion current.
- This is especially true for molecules with one or more basic nitrogen (or other basic) atoms. An exception to the general rule is that molecules with acidic sites, such as carboxylic acids and acid alcohols, produce more negative ions than positive ions.
- Although, in general, fewer negative ions are produced than positive ions, negative ion polarity is sometimes the mode of choice.
- This is because the negative ion polarity mode sometimes generates less chemical noise than does the positive mode. Thus, selectivity might be better in the negative ion mode than in the positive ion mode.

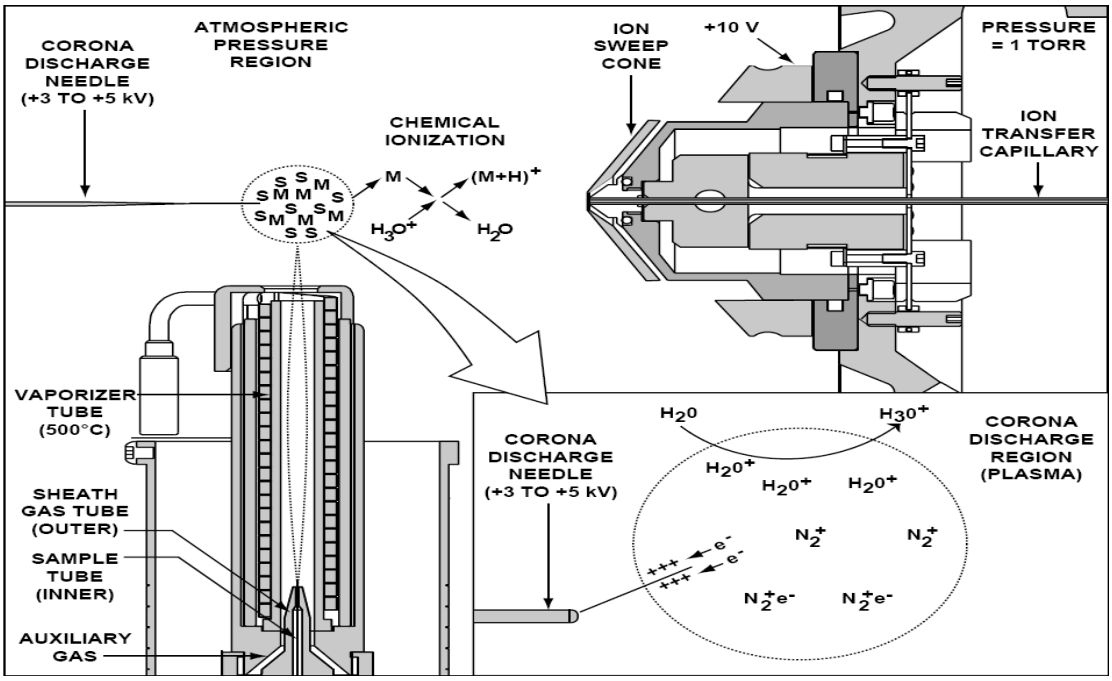


Figure 4.3 APCI process in the positive ion polarity mode

Scan modes:

- The mass spectrometer can be operated in a variety of scan modes. The most commonly used scan modes can be divided into two categories: single mass spectrometer scan modes and MS/MS scan modes. The scan modes in each category are as follows:
  - Mass spectrometer scan modes: Q1MS and Q3MS
  - MS/MS scan modes: Product, Parent, Neutral Loss
  - Data dependent scan mode
- The scan modes that can be employed depend on the number and type of rod assemblies and the voltages applied to the rod assemblies. The mass analyzer has three rod assemblies. The first and third rod assemblies, Q1 and Q3, are Hyper Quads (hyperbolic-profile quadrupoles), and the second rod assembly, Q2, is a square-profile quadrupole.
- Rod assemblies can be operated in either of two capacities:
  - Ion transmission devices
  - Mass analyzers
- If only RF voltage is applied, a rod assembly serves as an ion transmission device that passes all ions within a large range of mass-to-charge ratios (i.e., virtually all ions present).
- When both RF and DC voltages are applied to a rod assembly, the separation of ions of different mass-to-charge ratios occurs. This separation allows the rod assembly to serve as a mass analyzer.

- On the mass spectrometer, the hyper quad rod assemblies can be operated with RF and DC voltages or with only RF voltage. That is, Q1 and Q3 can act either as mass analyzers or ion transmission devices. The Q2 rod assembly always operates with only RF voltage. Thus, Q2 is always an ion transmission device. For a summary of how the rod assemblies function in several of the major scan modes,

Table 4.1 Different Scan Mode (MS & MS/MS)

Scan Mode	Q1 Hyperquad	Q2 Collision Cell	Q3 Hyperquad
Q1MS	Scan[a]	Pass all ions[b]	Pass all ions
Q3MS	Pass all ions	Pass all ions	Scan
Product	Set[d]	Fragment ions[c], then pass all fragments	Scan
Parent	Scan	Fragment ions, then pass all fragments	Set
Neutral Loss	Scan	Fragment ions, then pass all fragments	Scan

aScan = full scan or transmission of selected ions

bPass all ions or fragments = pass ions or fragments within a wide range of mass-to-charge ratios

cFragment ions = collisions with argon gas cause ions to fragment

dSet = set to pass ions of a single mass-to-charge ratio or a set of mass-to-charge ratios

Scan types:

- Mass spectrometer can be operated with a three scan types.
  - Full scan
  - Selected ion monitoring (SIM)
  - Selected reaction monitoring (SRM)

Full scan:

- The full scan type provides a full mass spectrum of each analyte. With full scan, the scanning mass analyzer is scanned from the first mass to the last mass, without interruption, in a given scan time.
- Full scan experiments are used to determine or confirm the identity of unknown compounds or the identity of each component in a mixture of unknown compounds.
- The full scan type gives you more information about an analyte than does SIM, but a full scan does not yield the sensitivity that can be achieved by the other two scan types. With full scan, you spend less time monitoring the signal for each ion than you do in SIM or SRM. Thus, full scan provides greater information but lower sensitivity than the other two scan types.

Selected ion monitoring:

- Selected ion monitoring (SIM) is a technique in which a particular ion or set of ions is monitored. SIM experiments are useful in detecting small quantities of a target compound in a complex mixture when the mass spectrum of the target compound is known. Thus, SIM is useful in trace analysis and in the rapid screening of a large number of samples for a target compound.
- Because only a few ions are monitored, SIM can provide lower detection limits and greater speed than the full scan modes. Lower detection limits are achieved because more time is spent monitoring significant ions that are known to occur in the mass spectrum of the target analyte. Greater speed is achieved because only a few ions of interest are monitored; regions of the spectrum that are empty or have no ions of interest are not monitored.
- SIM can improve the detection limit and decrease analysis time, but it can also reduce specificity. In SIM, only specific ions are monitored. Therefore, any compound that fragments to produce those ions will appear to be the target compound. Thus, a false positive result could be obtained.

Selected reaction monitoring:

- In selected reaction monitoring (SRM), a particular reaction or set of reactions, such as the fragmentation of an ion or the loss of a neutral moiety, is monitored.
- In SRM, a limited number of parent / product-ion pairs are monitored. In Product-type experiments, a parent ion is selected as usual, but generally only one product ion is monitored. SRM experiments are normally conducted with the product scan mode.
- Like SIM, SRM allows for the very rapid analysis of trace components in complex mixtures. However, because two sets of ions are being selected, the specificity obtained in SRM can be much greater than that obtained in SIM. Any interfering compound would not only have to form an ion source product (parent ion) of the same mass-to-charge ratio as the selected parent ion from the target compound, but that parent ion would also have to fragment to form a product ion of the same mass-to-charge ratio as the selected product ion from the target compound.

Data types:

- You can acquire and display mass spectral data (intensity versus mass-to charge ratio) with the mass spectrometer in one of two data types: Profile data type & Centroid data type

Profile data type:

- In the profile data type, you can see the shape of the peaks in the mass spectrum. Each atomic mass unit is divided into many sampling intervals. The intensity of the ion current is determined at each of the sampling intervals. The intensity at each sampling interval is displayed with the intensities connected by a continuous line. In general, the profile scan data type is used when you tune and calibrate the mass spectrometer so that you can easily see and measure mass resolution.

Centroid data type:

- In the centroid data type, the mass spectrum is displayed as a bar graph. In this scan data type, the intensities of each set of multiple sampling intervals are summed. This sum is displayed versus the integral center of mass of the sampling intervals. In general, the centroid scan data type is used for data acquisition because the scan speed is faster and the disk space requirements are smaller. Data processing is also much faster for centroid data.

4.1.2 TANDEM MASS SPECTROMETRY (MS/MS):

- All the mass spectrum tells us is the molecular weight, but it does not provide structure information for the component of interest. Efforts to remove this limitation took the form of the development of tandem mass spectrometric methods (MS/MS). Two stages of mass analysis are required, one to select the precursor ion from other ions generated in the ion source and one to analyze the product ions after collisions therefore this approach is called tandem mass spectrometry (MS/MS) [10, 11]. The schematic of tandem mass spectrometer is presented in Figure 4.4.
- In this technique ions are separated, identify and fragmented in a single instrument. A single molecular ion (precursor) that is characteristic of a given analyte is pass through a region where they are activated that causes them to fall apart to produce fragment ions (product). This is usually done by colliding the ions with a neutral gas in a process called collisional activation or collision-induced dissociation. It is a fast collision event, where ion translational energy is converted into ion internal energy to obtain an ion in the excited state, and slow unimolecular decomposition.
- The fragment ions are separated accordingly to their mass to charge ratio (m/z). The resulting MS/MS spectrum consists only of product ions from selected precursor. MS/MS can be performed in a number of single-stage instruments and a variety of two-stage instruments. The most versatile and most widely used MS/MS configuration is the triple quadrupole mass analyzer instrument as compared to other type of mass analyzer.

Recently different types of hybrid mass analyzers are developed for specific application for e.g. quadrupole-linear trap, quadrupole-time-of-flight; ion trap-time-of-flight.

- In triple quadrupole instruments mass analysis is performed in the first and third quadrupole while the second quadrupole is used as collision cell in the RF only mode. Different scan modes are available like product ion scan mode- for structure elucidation, parent ion scan mode and neutral scan mode -for screening, selected ion monitoring (SIM) and selected reaction monitoring (SRM) scan mode for quantitative analysis. The tandem mass spectrometry is a very specific and selective technique for quantitation of drug in different matrix.

4.1.3 LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS):

- By putting a separating chemistry from an HPLC in front of a specialized detector such as the mass spectrometer, one has a true multidimensional analytical technique that raises the level of the confidence of what you are looking at. Combining HPLC and MS offer the possibility of taking advantage of both HPLC as a powerful separation technique and MS as a powerful and sensitive detection and identification technique. HPLC chromatographic peaks may contain unresolved components that non-specific detectors cannot differentiate. This coupled with the need for more sensitive and specific detector for HPLC has generated considerable interest in the development of routine liquid chromatography-mass spectrometry (LC-MS)[37]. The mass spectrometer can be considered as a universal, selective and specific detector depending on selective mode of operation. An HPLC followed by mass spectrometric detection enables the quantification of therapeutically relevant drug concentration (µg/ml or ng/ml range) in small volume of biological samples and determinations of the chemical structures of biotransformation. The principle advantage of HPLC separation followed by the MS detection is that analyte molecular weight is identified by both chromatographic retention time and by molecular weight & fragmentation pattern. Two-dimensional separation is particularly important for the analysis of complex biological samples.
- The coupling between LC & MS has not been straight forward since the normal operation condition of both HPLC and MS are different high pressure/ high vacuum, low temperature/high temperature, liquid phase/ gas phase, high flow/low flow to achieve and to cope with these problem different LC-MS interface have been developed. The

constant limiting factor is the amount of liquid that can be introduced into the ion source of a MS prevented the wide spread development and use of LC-MS. But now many of the difficulties associated with it have been solved and there are now several interface designs and LC-MS is in routine use in analytical laboratories worldwide.

- The diversity of available interfaces: capillary inlet [12-14], pneumatic nebulizer interfaces [15], moving belt [16-18], direct liquid interface[19-21], thermospray [22], continuous - flow fast atom bombardment- FAB [23-25], particle beam [26,27]and atmospheric pressure ionization- API[28]. Currently API based LC/MS interfaces i.e. electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most widely approaches, while other has limited extent. A high electrical potential applied to a solvent emerging from a capillary causes the solvent to break into fine threads which disintegrate into small droplets, this phenomenon is called electrospray[28, 29].
- In APCI the column effluent is nebulized into a heated vaporizer tube and after desolvation the gas-vapour mixture enters the API source. In API source APCI is initiated by electrons generated at a corona discharge needle in combination with solvent vapours[28, 30]. More than 99% of LC-MS performed today is based on API interfacing. These interfaces in combination with tandem mass spectrometry of single stage and double stage fragmentation instrument enables a LC-MS/MS technique.
- ESI is one of the most exciting ionization methods due to direct ionization from solution and therefore its use as an LC-MS/MS interface, production of multiply charged ions that extends the effective mass range of the mass analyzer, introduction of methods to aid in desolvation of the analyte, low background from the ionization process are the advantages over other ionization. Extensions of the ESI interface led to miniaturized formats, microelectrospray and nanoelectrospray.
- The most important application area of LC-MS is in the pharmaceutical field, where LC-MS is involved in almost every step of drug development, testing and formulation. Because of its specificity and sensitivity LC-MS, especially in combination with LC-MS/MS has rapidly growing importance in other drug research area.
- LC-MS/MS is widely applied for drug development as well as routine analysis. In drug discovery activities like lead identification, lead optimization, in vitro and in vivo drug screening, preclinical activities like metabolite identification, impurity screening and degradant screening, clinical activities like quantitative bioanalysis and metabolite identification application of LC-MS and LC-MS/MS enhanced extensively.

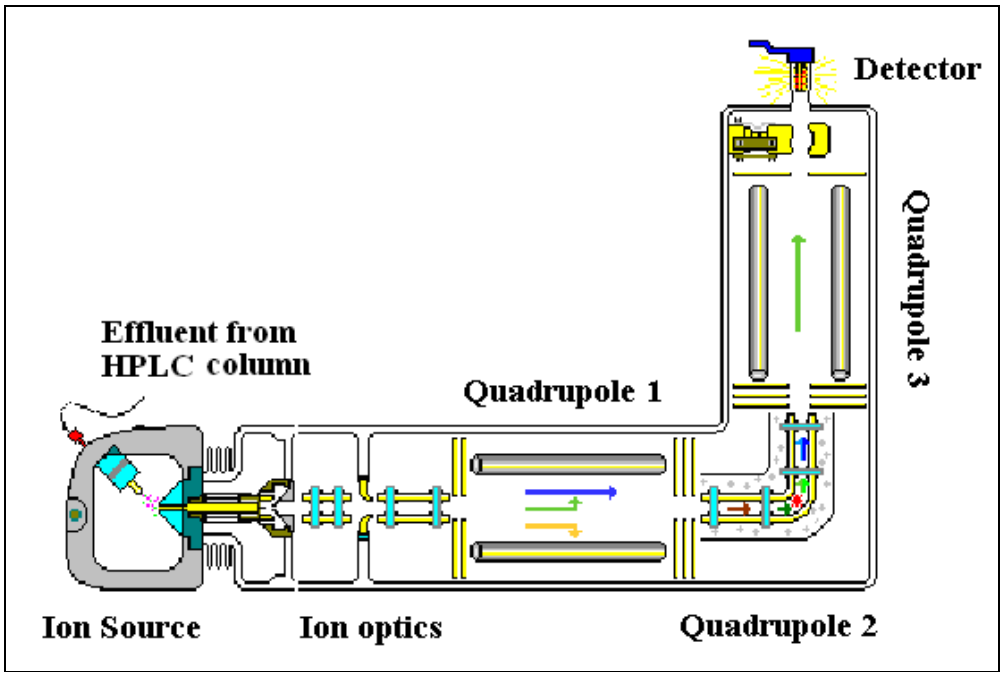


Figure 4.4 Schematic diagram of tandem mass spectrometer using triple quadrupole instrument.

4.2 EXPERIMENTAL WORK:

4.2.1 MATERIALS:

TABLE NO: 4.2 REFERENCE/WORKING STANDARDS

Sr. No.	Name of Standard	Batch no.	Assay	Name of manufacturer
1.	Gabapentin (Working standards)	GP0381107	99.97 %	Matrix Laboratories Ltd., Secunderabad
2.	Levetiracetam (Reference standards)	LR0520907	99.00 %	Hetero Labs Ltd., Hyderabad

TABLE NO: 4.3 APPARATUS

Apparatus	Grade/Capacity	Manufacturer/ Supplier
Volumetric flask	Appropriate volumes (Class A)	Borosil Glassworks Ltd., Mumbai
Glass beakers	Appropriate volumes	Borosil Glassworks Ltd., Mumbai
Glass bottles	Appropriate volumes	Borosil Glassworks Ltd., Mumbai
Solvent Filtration	Appropriate volumes	Millipore, India/ Borosil Glassworks Ltd., Mumbai
Measuring cylinder	Appropriate volumes (Class A)	Borosil Glassworks Ltd., Mumbai
Micropipette	Eppendorf, 2-20µl	Eppendorf India Ltd., Chennai
Micropipette	Eppendorf, 10-100µl	Eppendorf India Ltd., Chennai
Micropipette	Eppendorf, 20-200µl	Eppendorf India Ltd., Chennai
Micropipette	Eppendorf, 100-1000µl	Eppendorf India Ltd., Chennai
Microtube	MCT-175, 1.7ml clear	Axygen, Scientific, USA

TABLE NO: 4.4 INSTRUMENTATION

Instrument	Brand/Model	Manufacturer/ Supplier
Auto sampler	CTC PAL	CTC PAL Ltd., UK
Solvent delivery module	Accela pump	Thermo Finnigan Ltd., UK
MS Detector	TSQ Quantum ultra	Thermo Finnigan Ltd., UK
Vortexer	Spinix	Barnstead International, USA
Analytical balance	Mettler Toledo	Mettler Toledo India Pvt. Ltd., Mumbai
Solid Phase Extraction (SPE) Unit	Waters/ Orochem	Waters, USA/Orochem, India
Freezer (-70°C)	U725-86	New Brunswick Scientific co.inc., USA
Freezer (-20°C)	375 W	Vest frost, UK
Refrigerator(2-8°C)	RT34M	Samsung India Electronics Ltd., India
Ultrasonic bath	JAC 4020	Bio-sci instruments, India
Water purification system	Milli-Q	Millipore, USA
Vacuum Pump	Millipore	Millipore, India

TABLE NO: 4.5 REAGENTS AND CHEMICALS

Chemicals/Reagents	Grade	Manufacturer/ Supplier
Methanol	LCMS grade	Sigma-Aldrich, Germany
Water	Milli Q	Millipore, USA
Ammonium Formate	For Molecular biology	Sigma-Aldrich, Germany

4.2.2 PREPARATION OF SOLUTIONS:

a) Preparation of 5 mM Ammonium formate:

- Ammonium formate (315.30 mg) was weighed accurately and transferred in 1000 ml glass bottle. Water (about 1000 ml) was added using graduated cylinder and sonicated to dissolve.
- pH was adjusted to 3.0 with OPA.

b) Elution solution / Blank solution (Methanol 100 %):

c) Auto sampler rinsing solution (Methanol 100%):

d) Mobile phase preparation [Methanol: 5 mM Ammonium formate (80:20,% v/v)]

- In 1000 ml glass bottle, Methanol (800 ml) and 5mM Ammonium formate (200 ml) were added using graduated cylinder. Mixed well. The solution was filtered on a 0.45µm filter using solvent filtration apparatus by applying vacuum and sonicated it.

e) Preparation of diluent [Water : Methanol, (50: 50, % v/v)]:

- In 1000 ml glass bottle, water (500 ml) was taken using graduated cylinder. Methanol (500 ml) was added using graduated cylinder. Mixed well.

4.2.3 PREPARATION OF ANALYTE SOLUTIONS:

a) Preparation of GBP main stock solution (5.00 mg/ml):

- GBP working/reference standard (equivalent to 50 mg) was weighed accurately. Transferred it in 10 ml volumetric flask. Diluent (50 ml) was added and sonicated to dissolve. Diluted up to mark with diluent.

b) Preparation of GBP intermediate stock solutions:

1. GBP intermediate stock solution (2.50 mg/ml)

- Calculated volume of GBP main stock solution (5 ml) was transferred to 10 ml volumetric flask and diluted up to mark with diluent.

2. GBP intermediate stock solution (0.50 mg/ml)

- Calculated volume of above GBP intermediate stock solution (5 ml) was transferred to make conc. 0.50 mg/ml of GBP in 25 ml volumetric flask and diluted up to mark with diluent.

Note: a) Percentage purity/ assay of standard were considered while weighing.
b) Salt equivalency was considered while weighing.
c) Weight and volume of solution might be changed as per the requirement without altering the concentration for analysis.

4.2.4 PREPARATION OF INTERNAL STANDARD SOLUTION:

a) Preparation of LEV main stock solution (1.00 mg/ml):

- LEV working/reference standard (equivalent to 10 mg) was weighed accurately. Transferred it in 10 ml volumetric flask. Diluent (about 5.0 ml) was added and sonicated to dissolve. Diluted up to mark with diluent.

b) Preparation of LEV intermediate stock solution (0.20 mg/ml):

- The calculated volume of LEV main stock solution (2 ml) was transferred to make conc. 0.20 mg/ml in 10 ml volumetric flask and diluted up to mark with diluent.

c) Preparation of LEV spiking stock solution (1.50 µg/ml):

- The calculated volume of LEV intermediate stock solution (0.188 ml) was transferred to make conc. 1.50 µg/ml of LEV in 25 ml volumetric flask and diluted up to mark with diluent.

Note: a) Percentage purity/ assay of standard were considered while weighing.
b) Salt equivalency was considered while weighing.
c) Weight and volume of solution might be changed as per the requirement without altering the concentration for analysis.

4.2.5 PREPARATION OF CALIBRATION STANDARD AND QUALITY CONTROL SPIKING SOLUTIONS:

TABLE NO: 4.6

Volume of GBP intermediate stock solution (0.50 mg/ml) used (ml)	Diluent up to (ml)	Spiking stock solution	
		GBP Conc. (µg/ml)	Sample Name
0.02	10	1.00	CSS-01
0.04	10	2.00	CSS-02
0.12	10	6.00	CSS-03
0.24	10	12.00	CSS-04
0.48	10	24.00	CSS-05
0.96	10	48.00	CSS-06
1.44	10	72.00	CSS-07
1.92	10	96.00	CSS-08
2.40	10	120.00	CSS-09
0.06	10	3.00	LQCS
0.80	10	40.00	MQCS
1.80	10	90.00	HQCS
2.40	5	240.00	2ULOQCS

4.2.6 PREPARATION OF BIOLOGICAL MATRIX CALIBRATION AND QUALITY CONTROL SAMPLES IN MATRIX:

- The blank biological matrix (plasma) taken out from –20°C/-70°C deep freezer and kept at room temperature for thawing and vortexed adequately before pipetting.

a) Preparation of blank plasma:

- To analyte free plasma (95 µl) in micro tube diluent (5 µl) was added and vortexed to mix. Diluent (25 µl) was added and followed the sample treatment procedure as per section 4.2.12.

b) Preparation of zero standard:

- To analyte free plasma (95 µl) in micro tube diluent (5 µl) was added and vortexed to mix. Internal standard spiking stock solution (25 µl) was added and followed the sample treatment procedure as per section 4.2.12.

c) Preparation of calibration standards and quality control samples in biological matrix with respect to 5 % spiking:

TABLE NO: 4.7

Details of spiking stock solution used		Plasma volume (µl)	GBP Conc. (µg/ml)	Sample Name
GBP Conc. (µg/ml)	Volume (µl)			
1.00	5	95	0.05	CS-1
2.00	5	95	0.10	CS-2
6.00	5	95	0.30	CS-3
12.00	5	95	0.60	CS-4
24.00	5	95	1.20	CS-5
48.00	5	95	2.40	CS-6
72.00	5	95	3.60	CS-7
96.00	5	95	4.80	CS-8
120.00	5	95	6.00	CS-9
3.00	5	95	0.15	LQC
40.00	5	95	2.00	MQC
90.00	5	95	4.50	HQC
240.00	5	95	12.00	2ULOQ

d) System suitability:

- Prepared MQC level sample was used in system suitability and followed the sample treatment procedure as per section 4.2.12.

4.2.7 TUNING OF GBP AND LEV IN MS:

a) Preparation of tuning GBP solution (50 ng/ml):

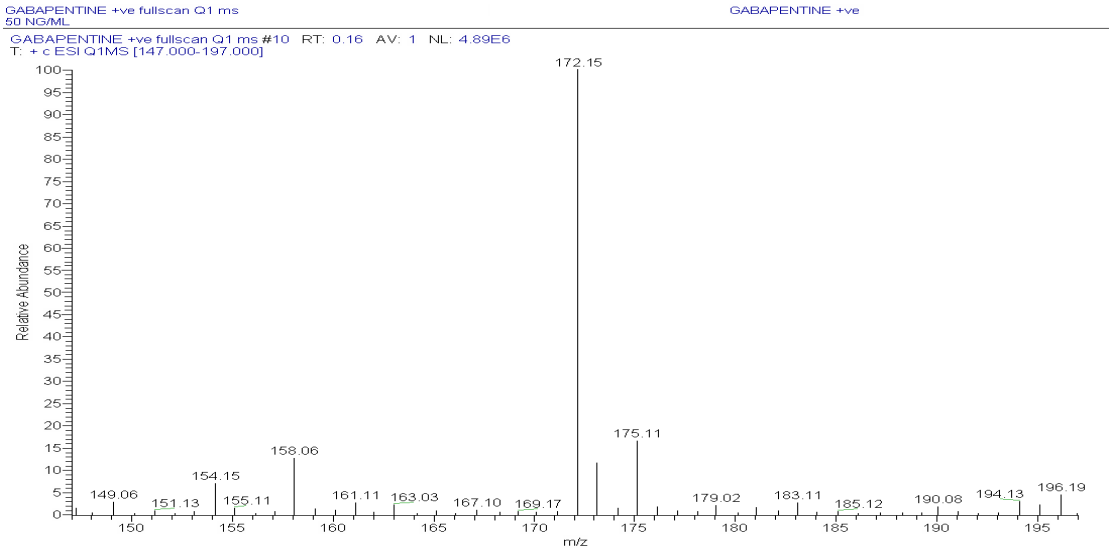
- GBP (5 mg) was taken in 10 ml vol. flask and made up the volume up to mark with methanol. Above solution (0.01 ml) was taken in 100 ml volumetric flask and diluted with methanol to 100 ml.

b) Preparation of tuning LEV solution (50 ng/ml):

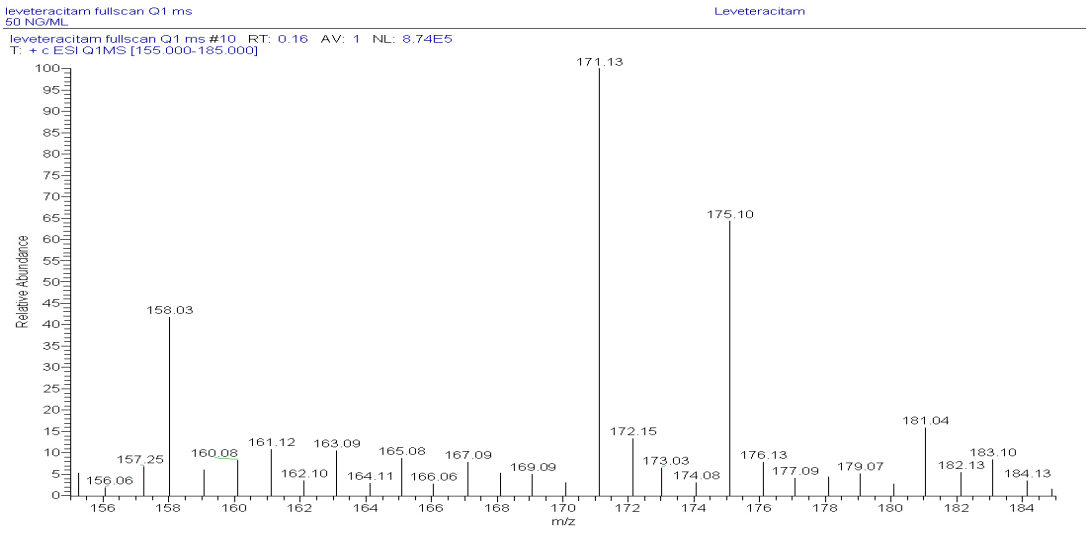
- LEV (5 mg) was taken in 10 ml vol. flask and made up the volume up to mark with methanol. Above solution (0.01 ml) was taken in 100 ml volumetric flask and diluted with methanol to 100 ml.

TABLE NO: 4.8 TUNING PARAMETERS FOR GBP & LEV (IS)

SR NO.	PARAMETERS	GBP	LEV
1.	Parent ion	172.15	171.13
2.	Product ion	154.15	126.06
3.	Collision energy	12 V	15 V
4.	Tube lens	78.59	66.83
5.	Q1pw	0.70	0.70
6.	Q3pw	0.70	0.70
7.	Spray voltage	4000 V	4000 V
8.	Spray capillary temp	390 °C	390 °C



GBP



LEV

Figure 4.5 Tuning Spectrum of GBP & LEV

4.2.8 METHOD DEVELOPMENT TRIALS:

TABLE NO: 4.9 METHOD DEVELOPMENT TRIALS

SR NO	TRIAL	COLUMN	MOBILE PHASE	SAMPLE	OBSERVATION
1.	TRIAL-1	Betabasic C8, 50 × 4.6 mm, 5 µ	ACN: 10 mM Amm. formate (80:20)	Aqueous MQC	peak shape was not good
2.	TRIAL-2	Betabasic C8, 50 × 4.6 mm, 5 µ	ACN: 5 mM Amm. formate (80:20)	Aqueous MQC	IS peak shape was not good
3.	TRIAL-3	Betabasic C8, 50 × 4.6 mm, 5 µ	ACN: 1 mM Amm. formate (80:20)	Aqueous MQC	peak shape was good , but higher tailing observed
4.	TRIAL-4	Betabasic C8, 50 × 4.6 mm, 5 µ	ACN: Methanol: 5 mM Amm. formate (60:20:20)	Aqueous MQC	peak shape was good , but higher tailing observed
5.	TRIAL-5	Betabasic C8, 50 × 4.6 mm, 5 µ	ACN: Methanol: 1 mM Amm. formate (60:20:20)	Aqueous MQC	peak shape was good, but higher tailing was observed
6.	TRIAL-6	Betabasic C8, 50 × 4.6 mm, 5 µ	ACN: Methanol: 1 mM Amm. formate (40:40:20)	Aqueous MQC	peak shape was good, slight higher tailing was observed
7.	TRIAL-7	Symmetry C8, 150 × 4.6 mm, 5 µ	ACN: Methanol: 1 mM Amm. formate (40:40:20)	Aqueous MQC	peak shape was good, slight less tailing was observed
8.	TRIAL-8	Symmetry C8, 150 × 4.6 mm, 5 µ	Methanol : 1 mM Amm. formate (80:20)	Aqueous MQC	peak shape was good, tailing was not observed
9.	TRIAL-9	Symmetry C8, 150 × 4.6 mm, 5 µ	Methanol : 1 mM Amm. formate (80:20)	Aqueous MQC & External spiked MQC	peak shape was good, ion suppression was observed

10.	TRIAL-10	Symmetry C8, 150 × 4.6 mm, 5 µ	Methanol : 5 mM Amm. formate (80:20)	Aqueous MQC & External spiked MQC	peak shape was good, ion suppression was not observed
11.	TRIAL-11	Symmetry C8, 150 × 4.6 mm, 5 µ	Methanol : 5 mM Amm. formate (80:20)	Linearity & P&A (Waters Cartridge)	Within acceptance criteria
12.	TRIAL-12	Symmetry C8, 150 × 4.6 mm, 5 µ	Methanol : 5 mM Amm. formate (80:20)	Linearity & P&A (Orochem Cartridge)	Within acceptance criteria

4.2.9 METHOD SUMMARY:

Biological matrix	:	Human plasma
Sample volume required	:	0.1 ml
Anticoagulant	:	Heparin
Analyte	:	Gabapentin
Internal standard	:	Levetiracetam
Calibration curve range	:	GBP : 0.05 µg/ml to 6.00 µg/ml
Detection mode	:	Mass spectrometry
Analytical technique	:	Liquid chromatography- Mass spectrometry
Sample treatment	:	Solid phase extraction
Quantitation method	:	Peak area ratio
Weighting factor for Calibration curve	:	$1/X^2$

4.2.10 CHROMATOGRAPHIC CONDITIONS:

Chromatographic mode	:	Reversed phase		
Isocratic/gradient mode	:	Isocratic		
Tray temperature	:	5°C		
Injection volume	:	5.0 µl		
Column	:	Brand	:	symmetry
	:	Type	:	C8

	:	Length x i.d. (mm)	:	150 x 4.6
	:	Particle size (µ)	:	5.0
Mobile phase	:	Methanol: 5 mM Ammonium formate (80:20, % v/v)		
Column Temp.	:	45°C		
Flow rate	:	1.0 ml/min with splitter. (30% to source & 70% to waste)		
Back pressure	:	140 bar (approximately)		
Retention times (min)	:	*		
GBP	:	1.66 min (± 0.5 min)		
LEV	:	1.66 min (± 0.5 min)		
Run time	:	3.00 min		

- Retention time of analyte and internal standard may vary between run/batch.

Shimadzu LC method parameters

Pump	:	Shimadzu LC-10ADvp
Pressure range	:	0- 410 Bars

System controller

Model	:	SIL-HTc
Rinse volume	:	1000 µl
Needle stroke	:	52 mm
Rinse speed	:	35 µL/sec
Purge time	:	1.0 minutes
Rinse dip time	:	0.0 sec
Rinse mode	:	Before and After aspiration.
Cooler enabled	:	Yes
Cooler temperature	:	5°C

4.2.11 DETECTOR PARAMETERS:

Ion source parameters :

Ion source	:	ESI mode
Spray voltage	:	4000 v
Sheath gas	:	55 (arb)
Auxiliary gas	:	30 (arb)
Capillary temperature	:	390 °C
Tube lens offset*	:	Tuned value 78.59 V (For GBP)
	:	Tuned value 66.83 V (For LEV)

Collision energy	:	12 V (For GBP), 15 V (For LEV)
Collision gas pressure	:	1.5 mTorr
MS acquire time	:	3.00 min.
No of segments	:	1
Segment time	:	3.00 min.
Chrom filter	:	10 sec
Peak width	:	0.01 for GBP & LEV.
No of scan events	:	2

Scan event – 1 (For GBP)

Polarity	:	Positive
Data type	:	Centroid
Scan type	:	SRM (Selected Reaction monitoring)

Parent	Product	Width	Time	CE	Q1PW	Q3PW
172.15	154.15	0.010	0.200	12	0.70	0.70

Scan event - 2 (For LEV)

Polarity	:	Positive
Data type	:	Centroid
Scan type	:	SRM (Selected Reaction monitoring)

Parent	Product	Width	Time	CE	Q1PW	Q3PW
171.13	126.06	0.010	0.200	15	0.70	0.70

Where,

Parent	:	Parent ion
Product	:	Daughter ion being monitored
Width	:	Scan width
Time	:	Scan time for event
CE	:	Collision energy
Q1PW	:	Resolution for first quadrupole
Q3PW	:	Resolution for third quadrupole
Divert Valve Settings	:	Not in use.

4.2.12 SAMPLE TREATMENT/SAMPLE PREPARATION:

- The study samples along with calibration standards and QC samples were taken out from the -20°C/-70°C deep freezer and kept at room temperature for thawing. Vortexed each sample adequately using a vortex mixer before pipetting. Followed the procedure from a to d as given below.

a) Internal standard spiking solution (25µl) was transferred in micro tube except in blank

plasma where 25 µl of diluent was added.

b) Plasma sample (100 µl) (blank plasma, zero standard, calibration standard, quality control samples) and study samples were added in micro tube using a micro pipette.

c) Water (100 µl) was added and vortexed to mix. The above plasma mixture was loaded on previously conditioned (conditioned with 1 ml of methanol and then with 1 ml of water) Orochem SPE columns (Orpheus, 30 mg, 1 ml, DVB-LP). The cartridges were washed with 1 ml of water and eluted with 1ml of elution solution & vortexed it.

d) Samples (5.0 µl) prepared as above was injected.

4.2.13 METHOD VALIDATION OF LC-MS/MS METHOD:

Pre method validation:

1. Pre method validation is a generic that provides concretes, statistical evidence for acceptable method performance prior to initiating a bioanalytical method validation.
2. A large number of replicate spiked matrix samples were analyzed in a single batch before method validation.
3. Pre method validation experiment was performed for **system suitability, specificity & selectivity, sensitivity, three linearity, three between-batches or inter-batch accuracy and precision, recovery and carry over effect check.**
4. All pre method validation samples prepared according to describe in sample preparation section.
5. The utility of this pre method validation approach was illustrated using actual laboratory data. The process of interpreting the results and drawing conclusions about assay viability was demonstrated.
6. The resulting conclusions provide sufficient background information to indicate if an assay procedure can be ready to enter in the validation process.

Method validation and characteristics of the Method:

A. Chromatography:

- Representative chromatograms of blank plasma, zero standard, LLOQ, ULOQ, LQC, MQC, and HQC samples GBP are represented in Figure No 4.6, 4.7, 4.8, 4.9, 4.10, 4.11 and 4.12, respectively.

B. Specificity and selectivity:

- Obtain samples of the relevant biological matrix (e.g., plasma, serum, blood, etc.) collected under controlled conditions.
- For plasma matrix, 08 normal plasma lots (04 different plasma lots with the anticoagulant to be used during method validation and 04 different plasma lots with the

anticoagulant to be used for study), 0l lipemic plasma and 01 haemolysed plasma lot were included.

- One sample each of the above mentioned 10 plasma lots at blank and LLOQ level was processed and analyzed as per the procedure described in sample preparation section.

Acceptance criteria:

- No interfering peaks from endogenous matrix components, decomposition product etc., should be present at the retention time of an analyte and an internal standard in blank matrix.
- If any peak is present at the retention time of analyte in blank matrix, its area response should be ≤20 % of response of an extracted lowest plasma calibration standard i.e. LLOQ standard of the same lot.
- If any peak is present at the retention time of an internal standard in blank matrix, its area response should be ≤5 % of the response of an extracted internal standard concentration of the same lot.
- At least 75 % of the buffered plasma and heparinised plasma should meet the acceptance criteria.
- Both lipemic and haemolysed plasma should meet the above three criteria for the interference at the respective retention time of analyte and internal standard. If the experiment fails, repeat the experiment and then change the methodology if required.

C. Sensitivity:

Procedure:

- Calibration standards, zero standard (matrix spiked only with internal standard) and six sets of matrix sample spiked at LLOQ concentration were processed and analyzed using blank matrix lot, as per the procedure described in sample preparation section.

Acceptance criteria:

- The analyte area response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte calculated concentration should be identifiable and reproducible with a precision of 20% and accuracy of 80- 120%

D. Linearity:

- A calibration curve (standard curve) is the relationship between the response of the instrument and known concentrations of the analyte.
- A calibration curve should be prepared for each analyte in the sample.
- A sufficient number of standards should be used in order to properly define the relationship between concentration and response.

- A calibration standard should be prepared in the same biological matrix same as that of the study samples, by spiking with known concentrations of the analyte.
- A calibration curve should be comprised of a "blank matrix" (matrix processed without analyte and internal standard), a "zero standard" (blank matrix processed only with internal standard) and six or more calibration standards covering the expected range, including the LLOQ and ULOQ.
- LLOQ should cover at least 4-5 half-life of the reported Cmax.
- ULOQ should cover the expected Cmax value. When no reference is available, it should be decided on basis of development studies results.
- Calibration curve must contain minimum six calibration standards. Selection of calibration standard expressed as multiple of LLOQ and percent of ULOQ are as follows:

TABLE NO: 4.10 SELECTION OF CALIBRATION STANDARD

A 8 Standard Calibration Curve	CODE	A 9 Standard Calibration Curve
LLOQ	Standard 1 – CS-1	LLOQ
5% ULOQ or 2 X LLOQ	Standard 2 – CS-2	2 X LLOQ
10% ULOQ	Standard 3 – CS-3	2% to 5% ULOQ
20% ULOQ	Standard 4 – CS-4	10% ULOQ
40% ULOQ	Standard 5 – CS-5	20% ULOQ
60% ULOQ	Standard 6 – CS-6	40% ULOQ
80% ULOQ	Standard 7 – CS-7	60% ULOQ
100% ULOQ	Standard 8 – CS-8	80% ULOQ
	Standard 9 – CS-9	100% ULOQ

Procedure:

- LLOQ and ULOQ calibration standards in duplicate (add suffix "D" in sample name of duplicate standard) were prepared, so that if first does not meet acceptance criteria, then only second standard of the same concentration level should be used in regression analysis. If both are within acceptance criteria consider the first standard for regression analysis.
- Blank matrix, zero standard and calibration standards were processed and analyzed as per the procedure described in sample preparation section.
- Summary of the curve fitting parameters and of back-calculated concentration at each standard level from each calibration curve were prepared.
- % nominal concentration of back-calculated value at each calibration level to determine accuracy at each calibration level was calculated.

Acceptance criteria:

- A correlation coefficient (r) of the calibration curve must be $\geq$ 0.9900.
- The back-calculated concentration of the lowest calibration standard (CS1) must be within 80-120% of its theoretical concentration.
- The back-calculated concentrations of all other calibration standards must be within 85-115% of their theoretical concentrations.
- The curve must contain at least 75% of the calibration standards for evaluation of curve fitting.
- No two adjacent (or consecutive) calibration standards can be rejected.
- Both replicates each of LLOQ and ULOQ standard cannot be rejected, either of the two replicate must meet the acceptance criteria.

E. Accuracy:

1. Within-batch or intra-batch accuracy:

- Calibration standards and six replicates each of LLOQ, LQC, MQC and HQC samples were processed and analyzed as per the procedure described in sample preparation section.
- % nominal concentration of back-calculated value for LLOQ, LQC, MQC and HQC, analyzed in single analytical batch was calculated, as per formula to determine within-batch or intra-batch accuracy.

2. Between -batch or inter-batch accuracy:

- Calibration standards and six replicates each of LLOQ, LQC, MQC and HQC samples were processed and analyzed as per the procedure described in sample preparation section.
- Five different batches on different days were performed.
- % nominal concentration of back-calculated value for LLOQ, LQC, MQC and HQC samples, analyzed on five different batches on different days was calculated.

Acceptance criteria:

- The back calculated concentrations of all QC samples (LQC, MQC, and HQC) must be within 85-115% of their nominal concentration except at LLOQ sample where it should not deviate by more than 80-120% of its nominal concentration.
- At least 67% quality control samples must fall within above-mentioned criteria at each LLOQ, LQC, MQC, and HQC levels.

F. Precision:

1. Within-batch or intra-batch precision:

- Calibration standards and six replicates each of LLOQ, LQC, MQC and HQC samples were processed and analyzed as per the procedure described in sample preparation section.
- Mean, standard deviation and % coefficient of variation for LLOQ, LQC, MQC and HQC samples, analyzed in single analytical batch were calculated.

2. Between -batch or inter-batch precision:

- Calibration standards and six replicates of LLOQ, LQC, MQC and HQC samples were processed and analyzed as per the procedure described in sample preparation section.
- Five different batches on different days were performed.
- Mean, standard deviation and % coefficient of variation for LLOQ, LQC, MQC and HQC samples analyzed on five different batches on different days were calculated.

Acceptance criteria:

- The %CV of the back-calculated concentrations of all QC samples (LQC, MQC, and HQC) must be within 15%, except for LLOQ, which should be within 20%.
- At least 67% quality control samples must fall within above-mentioned criteria at each LLOQ, LQC, MQC and HQC levels.

G. Recovery:

- Recovery for analyte and internal standard was performed by comparing the area of extracted samples at three different concentrations (LQC, MQC and HQC) with un-extracted standards area that represents 100% recovery.

Procedure:

- Recovery of an analyte was determined at LQC, MQC, HQC levels and recovery of an internal standard was determined at concentration to be used during method validation.
- Six replicates each of LQC, MQC, and HQC samples were processed and analyzed as per the procedure described in sample preparation section. These were considered as extracted samples for recovery.
- To prepare comparison samples for recovery, extracted solutions of blank matrix were used, for the preparation of equivalent unextracted samples, to nullify the matrix effect while calculating the recovery.
- For the recovery comparison samples, the spiking stock of standard solution of an analyte and internal standard were spiked in the extracted blank matrix solution to achieve concentration equivalent to extracted samples nominal concentration.

- Unextracted standard solution of an analyte (at LQC, MQC and HQC level) and internal standard of concentrations equivalent to concentration of extracted samples were processed and analyzed.
- The area for extracted LQC, MQC, HQC samples and internal standard were compared with areas of unextracted standards and internal standard that represent 100 % recovery.
- % recovery of an analyte at LQC, MQC and HQC samples and an internal standard were calculated.
- % recovery for extracted HQC, MQC and LQC samples should not exceed 115%, if it exceeds then reporting should be justified.

Acceptance criteria:

- Recovery of analyte and internal standard should be consistent, precise and reproducible.
- Variability within areas at each QC level for analyte should be within CV of 15%.
- Variability of the analyte recoveries across all the three QC levels should be within C.V of 20%.
- Variability within areas of IS found with each extracted QC level should be within CV of 15%, while the variability of internal standard area found between all the QC levels should be within CV of 20%.

H. Dilution integrity:

Procedure:

- The stock solutions of analyte and internal standard were prepared as per the procedure described in sample preparation section.
- Analyte spiking stock solution was spiked in blank plasma to get concentration equivalent to 2 times of ULOQ.
- Above spiked samples (2xULOQ) was diluted with blank plasma to get 1/2 and 1/4 concentrations of the spiked sample or as per requirement.
- Calibration standards and six aliquots each of diluted samples (1/2 and 1/4 dilutions) were processed and analyzed as per the procedure as described in sample preparation section.
- %CV for the back calculated concentration was calculated.
- % accuracy for the back calculated concentration was calculated.

Acceptance criteria:

- Back calculated concentration accuracy should be within 85-115% of theoretical concentration.
- Precision (% CV) for the back calculated concentration should be within 15%.

- At least 67% of total dilution samples at each dilution level should fall within above-mentioned criteria.

I. Matrix effect:

Procedure:

- Calibration standards were processed and analyzed in the same matrix which was to be used during validation experiment and three replicates from all lots described above each at LQC and HQC levels were processed and analyzed as per the procedure described in **s**ample preparation section.

Acceptance criteria:

- The back calculated concentrations of LQC and HQC must be within 85- 115% of their theoretical concentration.
- At least 67% of LQC and HQC samples must fall within above-mentioned criteria for each lot of matrix.
- At least 75% of the buffered plasma should meet the acceptance criteria.
- Both lipemic and haemolysed plasma should meet the above criteria, if the experiment fails repeat the experiment once or change the methodology.

J. Stability:

1. Stock solution stability:

Procedure:

- Fresh stock solution of an analyte and an internal standard were prepared as per the procedure described in sample preparation section.
- Freshly prepared main stock solutions aliquots of analyte and internal standard solution were stored at 2-8°C in refrigerator or in freezer if required for a relevant period for short-term and long-term stability.
- Freshly prepared main stock solutions aliquots of analyte and internal standard solution were stored at room temperature for at least 6 hrs or relevant short-term period.
- After relevant stability period fresh stock comparison solution of analyte and an internal standard were prepared as per the procedure.
- The main stock aliquots were retrieved from refrigerator and from room temperature.
- Appropriately stock solution of analyte was diluted to get concentration equivalent to ULOQ nominal concentration for both comparison and stability sample solution.
- Six injections of freshly prepared diluted analyte and internal standard comparison solution and stability solutions were performed.
- The analytical area results of stability solutions were compared with those of freshly prepared solutions area results.

- After relevant long-term stability period at 2-8°C, fresh stock comparison solution of analyte and an internal standard were prepared as per the procedure.
- Above steps were repeated for long-term solution stability.
- The area results of long term stability solutions were compared with those of freshly prepared comparison solutions.
- % mean change for analyte and internal standard was calculated.

Acceptance criteria:

- % mean change between freshly prepared stock solution (comparison samples) area results and stability samples area results must be within ± 10%.
- If %CV of area of analyte and internal standard for both stability and comparison sample is greater than 5 % than out of six only one inappropriate sample should be rejected.
- If above acceptance criteria are not met, then experiment should be repeated if on investigation any processing error is found or should be repeated for relevant shorter storage period or storage temperature.

Main stock solution stability of GBP and LEV (IS) at room temperature for 93 hrs:

- Main stock solution of GBP and LEV (IS) were freshly prepared and aliquots of stocks were kept at room temperature for 93 hrs (stability sample). Aqueous equivalent highest calibration standards of GBP and LEV (IS) were prepared from the stability samples and analyzed. Areas of stability samples and freshly prepared samples were compared to determine % mean change during stability period.

Main stock solution stability of GBP and LEV (IS) at 2-8°C for 93 hrs:

- Main stock solution of GBP and LEV (IS) were freshly prepared and aliquots of stocks were kept at 2-8°C for 93 hrs (stability sample). Aqueous equivalent highest calibration standards of GBP and LEV (IS) were freshly prepared from the stability samples and analyzed. Areas of stability samples and freshly prepared samples were compared to determine % mean change during stability period.

2. Bench top stability of GBP (at room temperature for 20 hrs):

Procedure:

- Six aliquots each of LQC and HQC samples were spiked for bench top stability. These samples were kept at room temperature for 20 hrs.
- In case of spiking and keeping the bench top stability samples at room temperature, the bench top stability period was considered between the time of keeping at room temp. and the time of start of processing those samples.
- Calibration standards and six replicates each of LQC and HQC comparison samples prepared by spiking with freshly prepared stock solutions or stock solutions with proven

stability were processed and analyzed as per the procedure described in sample preparation section.

- The stability samples after relevant period (at room temperature for 20 hrs) were processed and analyzed.
- The mean back-calculated concentration of stability samples were compared with those of freshly prepared comparison samples.
- % mean change as per formula was calculated.

Acceptance criteria:

- The back-calculated concentrations of all LQC and HQC samples must be within 85-115% of their theoretical concentration.
- At least 67% QC samples must fall within above-mentioned criteria at each LQC and HQC levels.
- % Mean change must be within ± 15%.

3. Process stability of GBP at 5°C in auto sampler for 76 hrs:

- Process stability of analyte was determined at LQC and HQC levels.

Procedure:

- Calibration standards and six replicates each of LQC and HQC comparison samples prepared by spiking with freshly prepared stock solutions were processed and analyzed as per the procedure described or use the stored samples.
- The above QC samples were kept in the auto sampler for relevant period (at 5°C for 76 hrs) to measure the stability of the processed samples in the auto sampler.
- Stability samples after relevant period along with calibration standards and six replicates each of LQC and HQC comparison samples prepared by spiking with freshly prepared stock solutions or stock solutions with proven stability were processed and analyzed as per the procedure.
- The process stability period was considered between the time of storing of samples in auto sampler or refrigerator and the time of analysis of last process stability sample.
- The mean back-calculated concentration of stability samples with those of freshly prepared comparison samples was calculated.
- % mean change as per formula was calculated.

Acceptance criteria:

- The back-calculated concentrations of all LQC and HQC samples must be within 85-115% of their nominal concentration.
- At least 67% of quality control samples must fall within above-mentioned criteria at each LQC and HQC levels.

- % Mean change must be within ± 15%.

4. Freeze and thaw stability of GBP (after 4th cycle at -20°C and -70°C):

- Freeze and thaw stability of analyte was determined after three freeze and thaw cycles at LQC and HQC levels.

Procedure:

- Three sets (set-1, set-2 and set-3) each having six replicates each of LQC and HQC sample of freeze and thaw stability by spiking with freshly prepared stock solutions or stock solutions with proven stability were prepared.
- All the samples were stored at –20°C and/or –70°C for at least 24 hrs and after this period all the three sets of samples were thawed unassisted at room temperature.
- When completely thawed, set-1, set-2 and set-3 were refrozen for at least 12 hrs under the same conditions.
- Kept set-1 in frozen condition to use for second freeze and thaw cycle when analytical results of third freeze and thaw cycle were not within the acceptance criteria.
- The second freeze - thaw cycle was repeated for set-2 and set-3.
- The frozen set-2 and set-3 were thawed for the third freeze and thaw cycle. Set-3 was thawed.
- Calibration standards and six replicates each LQC and HQC comparison samples were prepared by spiking with freshly prepared stock solutions or stock solution with proven stability as per the procedure. Those samples were processed and analyzed.
- Set-2 stability samples after third freeze and thaw cycle along with freshly prepared low and high quality control comparison samples were processed and analyzed.
- Fourth freeze and thaw cycle for set-3 was repeated.
- The mean back-calculated concentration of stability samples with those of freshly prepared comparison samples was calculated.
- % mean change was calculated as per formula.

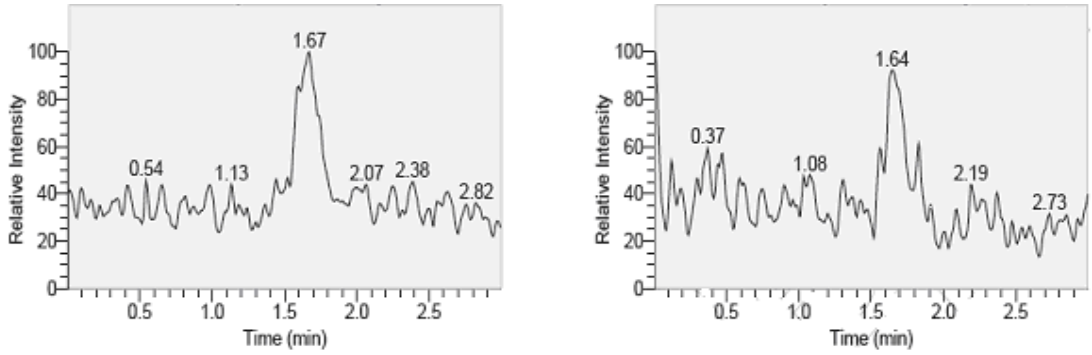
Acceptance criteria:

- The back-calculated concentrations of all LQC and HQC samples must be within 85- 115 % of their nominal concentration.
- At least 67 % of QC samples must fall within above-mentioned criteria at each LQC and HQC levels.
- % Mean change must be within ± 15 %.

4.3 RESULTS AND DISCUSSION:

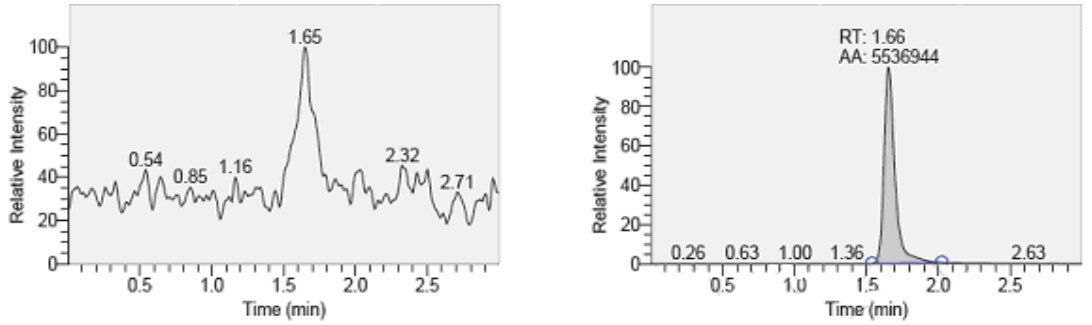
4.3.1 Chromatography:

- Representative chromatograms of blank plasma, zero standard, LLOQ, ULOQ, LQC, MQC, and HQC samples GBP are represented in Figure No. 4.6, 4.7, 4.8, 4.9, 4.10, 4.11 and 4.12, respectively.



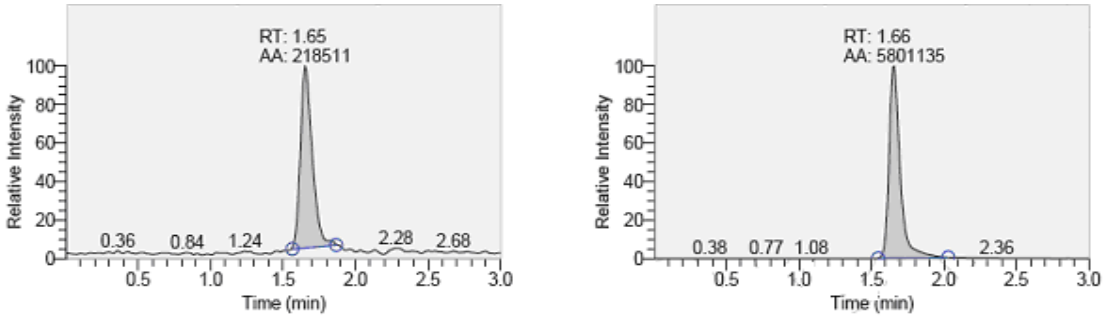
GBP **LEV**

Figure 4.6 Representative chromatogram of blank plasma



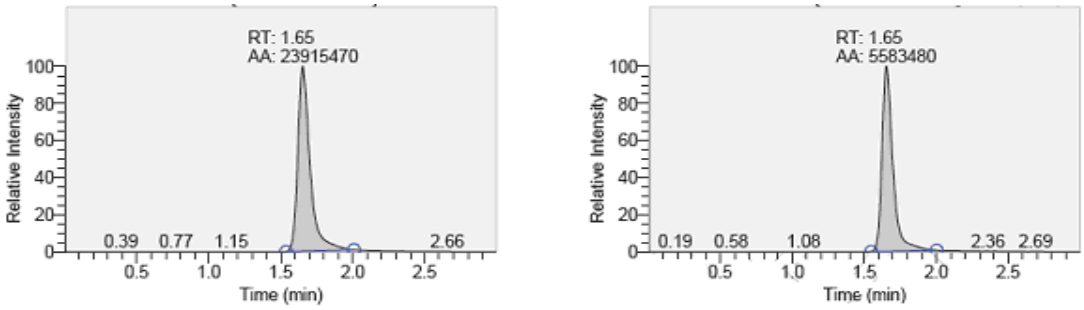
GBP **LEV**

Figure 4.7 Representative chromatogram of zero standard



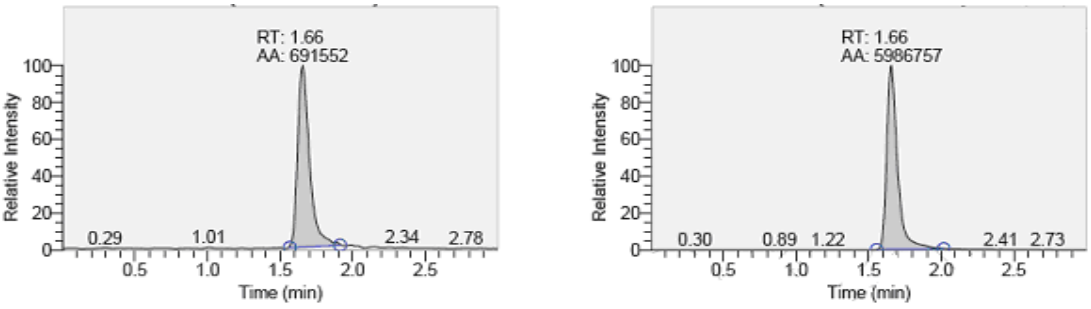
GBP **LEV**

Figure 4.8 Representative chromatogram of LLOQ



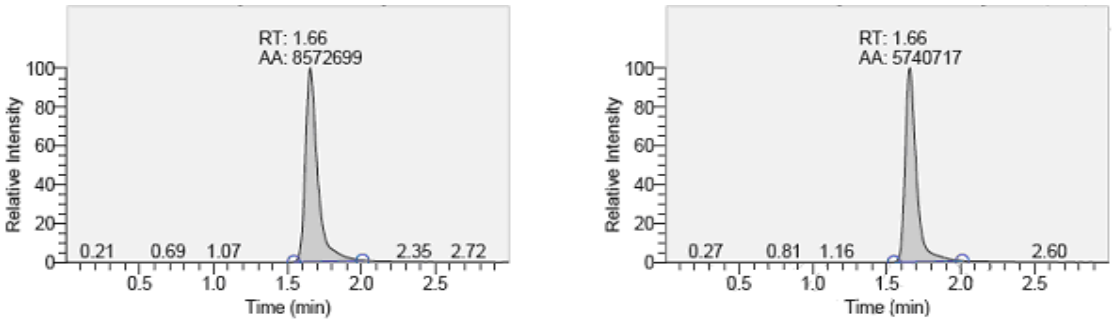
GBP **LEV**

Figure 4.9 Representative chromatogram of ULOQ



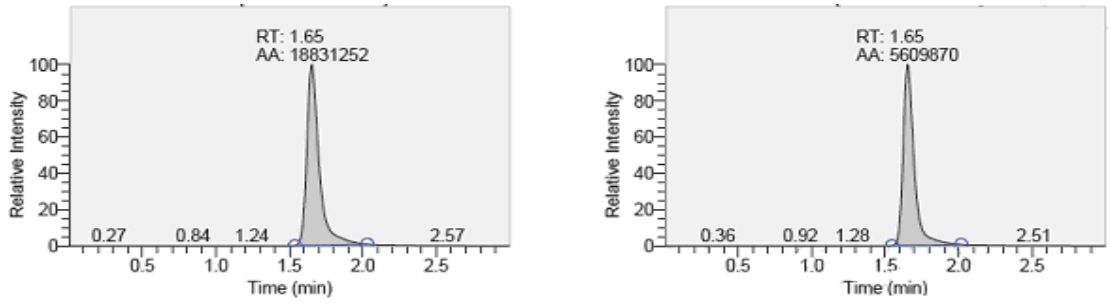
GBP **LEV**

Figure 4.10 Representative chromatogram of LQC



GBP **LEV**

Figure 4.11 Representative chromatogram of MQC



GBP **LEV**

Figure 4.12 Representative chromatogram of HQC

4.3.2 Specificity and selectivity:

- Four different lots of heparinised plasma, one lot of lipemic plasma and one lot of heamolysed plasma were chromatographed and no area response observed at the RT of GBP and no area response at the RT of IS (Table No. 4.11).

TABLE NO: 4.11
SPECIFICITY AND SELECTIVITY OF BLANK HUMAN PLASMA FOR GBP AND LEV

Sr. No.	Sample Name	Area		% of area	
		GBP	LEV	GBP	LEV
1	BLANK PLASMA(B225-08)	NF	NF	0.0	0.0
2	LLOQ(B225-08)	161694	4789031	NA	NA
3	BLANK PLASMA(B233-08)	NF	NF	0.0	0.0
4	LLOQ(B233-08)	156619	4865603	NA	NA
5	BLANK PLASMA(B235-08)	NF	NF	0.0	0.0
6	LLOQ(B235-08)	168187	4516163	NA	NA
7	BLANK PLASMA(B257-08)	NF	NF	0.0	0.0
8	LLOQ(B257-08)	155397	4764312	NA	NA
9	BLANK PLASMA(C001-08)	NF	NF	0.0	0.0
10	LLOQ(C001-08)	161691	4727598	NA	NA
11	BLANK PLASMA(D001-08)	NF	NF	0.0	0.0
12	LLOQ(D001-08)	155965	4653627	NA	NA

NA: Not Applicable, NF: Not Found

4.3.3 Sensitivity:

- The LLOQ was 0.05 µg/ml for GBP. The % CV of GBP at LLOQ was found to be 9.60. The % nominal concentration for LLOQ samples of GBP was 96.33 (Table No.4.12).

TABLE NO: 4.12
SENSITIVITY (LLOQ OF GBP)

Sr. No.	Sample Name	Conc.	Area		Area Ratio	Calculated	% Nominal conc.
		(µg/ml)	GBP	LEV		Conc. (µg/ml)	
1	LLOQ	0.05	196834	5711167	0.034	0.049	98.00
2	LLOQ	0.05	152127	5403757	0.028	0.040	80.00
3	LLOQ	0.05	195501	5744727	0.034	0.049	98.00
4	LLOQ	0.05	187094	5648930	0.033	0.047	94.00
5	LLOQ	0.05	200096	5738419	0.035	0.050	100.0
6	LLOQ	0.05	218511	5801135	0.038	0.054	108.0
n						6	
Mean						0.048	96.33
SD						0.005	
%CV						9.60	

4.3.4 Linearity:

- The Linearity of the method was determined by a weighted least square linear regression analysis of standard plots associated with a nine-point standard calibration curve. Best-fit calibration curves of peak area ratio versus concentration were drawn. The calibration curves were linear from 0.05 µg/ml to 6.00 µg/ml with correlation coefficient of $r^2 \geq 0.9976$ for GBP (Table No. 4.13 and 4.14, Figure 4.13).

TABLE NO: 4.13
SUMMARY OF CALIBRATION CURVE PARAMETERS OF GBP

Sr. No.	SLOPE	INTERCEPT	r	r^2
1	0.7104	-0.0004	0.9995	0.9991
2	0.7685	0.0020	0.9997	0.9994
3	0.6659	0.0033	0.9997	0.9994
4	0.6993	0.0029	0.9988	0.9976
5	0.7235	0.0029	0.9988	0.9976

TABLE NO: 4.14
BACK CALCULATED CONCENTRATION OF CALIBRATION STANDARDS FROM CALIBRATION CURVE OF GBP

LINEARITY		Conc. (µg/ml)								
Sr. No.		CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9
		0.05	0.10	0.30	0.60	1.20	2.40	3.60	4.80	6.00
1	% Nominal	0.049	0.104	0.293	0.578	1.205	2.331	3.715	4.905	6.030
	Deviation	-2.00	4.00	-2.33	-3.67	0.42	-2.88	3.19	2.19	0.50
2	% Nominal	0.050	0.101	0.294	0.609	1.153	2.392	3.708	4.802	6.075
	Deviation	0.00	1.00	-2.00	1.50	-3.92	-0.33	3.00	0.04	1.25
3	% Nominal	0.050	0.100	0.293	0.592	1.189	2.378	3.750	4.747	6.100
	Deviation	0.00	0.00	-2.33	-1.33	-0.92	-0.92	4.17	-1.10	1.67
4	% Nominal	0.051	0.095	0.281	0.640	1.177	2.349	3.762	4.775	6.093
	Deviation	2.00	-5.00	-6.33	6.67	-1.92	-2.12	4.50	-0.52	1.55
5	% Nominal	0.051	0.096	0.283	0.654	1.190	2.373	3.707	4.777	5.864
	Deviation	2.00	-4.00	-5.67	9.00	-0.83	-1.12	2.97	-0.48	-2.27
Mean		0.050	0.099	0.289	0.615	1.183	2.365	3.728	4.801	6.032
SD		0.001	0.003	0.006	0.029	0.017	0.022	0.023	0.055	0.088
%CV		2.00	3.03	2.08	4.72	1.44	0.93	0.62	1.15	1.46
% Nominal conc.		100.0	99.00	96.33	102.5	98.58	98.54	103.5	100.0	100.5

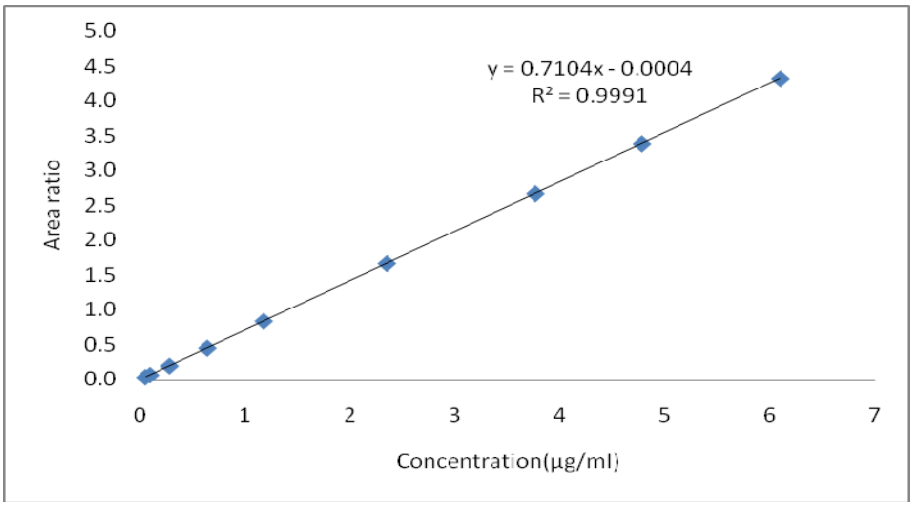


Figure 4.13 Representative calibration curve of GBP

4.3.5 Accuracy:

Within-batch or intra-batch accuracy:

- The % nominal concentration for LLOQ, LQC, MQC and HQC samples of GBP were found 96.33, 105.0, 104.9 and103.9, respectively (Table No. 4.15).

Between -batch or inter-batch accuracy:

- The % nominal concentration for LLOQ, LQC, MQC and HQC samples of GBP were found 99.00, 101.7, 101.4 and 101.6, respectively (Table No. 4.16).

4.3.6 Precision:

Within-batch or intra-batch precision:

- The % CV for LLOQ, LQC, MQC and HQC samples of GBP were 9.60, 4.54, 2.11 and 2.14, respectively (Table No. 4.15).

Between -batch or inter-batch precision:

- The % CV for LLOQ, LQC, MQC and HQC samples of GBP were 7.90, 3.38, 4.88 and 2.73, respectively. (Table No. 4.16).

TABLE NO: 4.15
WITHIN-BATCH OR INTRA-BATCH ACCURACY AND PRECISION OF GBP

Sr. No.	LLOQ (0.05 µg/ml)		LQC (0.15 µg/ml)	
	Calculated conc.	% Nominal conc.	Calculated conc.	% Nominal conc.
1	0.049	98.00	0.163	108.7
2	0.040	80.00	0.166	110.7
3	0.049	98.00	0.154	102.7
4	0.047	94.00	0.162	108.0
5	0.050	100.0	0.148	98.67
6	0.054	108.0	0.152	101.3
n	6		6	
Mean	0.048	96.33	0.158	105.0
SD	0.005		0.007	
%CV	9.60		4.54	
Sr. No.	**MQC (2.00 µg/ml)**		**HQC (4.50 µg/ml)**	
	Calculated conc.	% Nominal conc.	Calculated conc.	% Nominal conc.
1	2.103	105.1	4.726	105.0
2	2.172	108.6	4.803	106.7
3	2.092	104.6	4.619	102.6
4	2.094	104.7	4.734	105.2
5	2.088	104.4	4.636	103.0
6	2.034	101.7	4.523	100.5
n	6		6	
Mean	2.097	104.9	4.674	103.9
SD	0.044		0.100	
%CV	2.11		2.14	

TABLE NO: 4.16
BETWEEN-BATCH OR INTER-BATCH ACCURACY AND PRECISION OF GBP

Sr. No.	Day	LLOQ (0.05 µg/ml)		LQC (0.15 µg/ml)	
		Calculated conc.	% Nominal conc.	Calculated conc.	% Nominal conc.
1	Day 1	0.049	98.00	0.163	108.7
2		0.040	80.00	0.166	110.7
3		0.049	98.00	0.154	102.7
4		0.047	94.00	0.162	108.0
5		0.050	100.0	0.148	98.67
6		0.054	108.0	0.152	101.3
7	Day 2	0.054	108.0	0.152	101.3
8		0.049	98.00	0.148	98.67
9		0.053	106.0	0.158	105.3
10		0.049	98.00	0.154	102.7
11		0.046	92.00	0.158	105.3
12		0.051	102.0	0.156	104.0
13	Day 3	0.063	126.0	0.146	97.33
14		0.052	104.0	0.151	100.7
15		0.049	98.00	0.155	103.3
16		0.051	102.0	0.154	102.7
17		0.044	88.00	0.149	99.33
18		0.050	100.0	0.156	104.0
19	Day 4	0.049	98.00	0.147	98.00
20		0.049	98.00	0.149	99.33
21		0.049	98.00	0.149	99.33
22		0.047	94.00	0.145	96.67
23		0.048	96.00	0.151	100.7
24		0.045	90.00	0.146	97.33
25	Day 5	0.052	104.0	0.147	98.00
26		0.050	100.0	0.150	100.0
27		0.052	104.0	0.153	102.0
28		0.046	92.00	0.153	102.0
29		0.048	96.00	0.153	102.0
30		0.050	100.0	0.152	101.3
n		30		30	
Mean		0.050	99.00	0.153	101.7
SD		0.004		0.005	
%CV		7.90		3.38	

Table No. 4.16 continue

Sr. No.	Day	MQC (2.00 µg/ml)		HQC (4.50 µg/ml)	
		Calculated conc.	% Nominal conc.	Calculated conc.	% Nominal conc.
1	Day 1	2.103	105.1	4.726	105.0
2		2.172	108.6	4.803	106.7
3		2.092	104.6	4.619	102.6
4		2.094	104.7	4.734	105.2
5		2.088	104.4	4.636	103.0
6		2.034	101.7	4.523	100.5
7	Day 2	2.108	105.4	4.597	102.2
8		2.113	105.7	4.579	101.7
9		2.075	103.8	4.751	105.6
10		1.806	90.30	4.632	102.9
11		2.023	101.2	4.664	103.6
12		1.819	90.91	4.596	102.1
13	Day 3	2.091	104.6	4.536	100.8
14		2.049	102.5	4.583	101.8
15		1.806	90.30	4.610	102.4
16		1.995	99.75	4.567	101.5
17		1.788	89.40	4.627	102.8
18		2.062	103.1	4.476	99.47
19	Day 4	2.083	104.2	4.477	99.49
20		2.089	104.5	4.535	100.8
21		2.095	104.8	4.458	99.07
22		2.072	103.6	4.587	101.9
23		1.996	99.8	4.745	105.4
24		2.038	101.9	4.655	103.4
25	Day 5	2.080	104.0	4.407	97.93
26		2.051	102.6	4.418	98.18
27		2.015	100.8	4.275	95.00
28		2.038	101.9	4.346	96.58
29		1.953	97.65	4.513	100.3
30		2.018	100.9	4.415	98.11
n				30	
Mean		2.028	101.4	4.570	101.6
SD		0.099		0.125	
%CV		4.88		2.73	

4.3.7 Recovery:

- The percentage recovery of GBP was determined by comparing the mean peak area of GBP in extracted LQC, MQC and HQC samples with freshly prepared Unextracted LQC, MQC and HQC samples, respectively.
- The mean % recovery for LQC, MQC and HQC samples of GBP were 75.16, 74.24 and 74.48, respectively. (Table No. 4.17).
- For LEV (IS), mean peak area of eighteen extracted samples was compared to the mean area peak of eighteen Unextracted LEV (IS) samples. The mean percentage recovery for LEV (IS) was 104.5 (Table No.4.19).
- The % CV Unextracted for LQC, MQC and HQC samples of GBP were 1.37, 0.84 and 1.38, respectively. (Table No. 4.17).

- The % CV Extracted for LQC, MQC and HQC samples of GBP were 1.69, 2.20 and 1.95, respectively. (Table No. 4.17).
- The % CV of recovery across QC level for GBP was 0.64 (Table No. 4.18).
- The % CV within LEV IS concentration of Unextracted and Extracted sample area across QC level was 1.92 and 2.65, respectively (Table No.4.19).

TABLE NO: 4.17
RECOVERY OF GBP

Sr. No.	LQC (0.15 µg/ml) Area of GBP	
	Unextracted	Extracted
1	875947	684127
2	899832	667856
3	904803	676503
4	889850	662878
5	877122	659813
6	880627	653359
n	6	6
Mean	888030.17	667422.67
SD	12196.07	11296.22
%CV	1.37	1.69
%Recovery	75.16	
Sr. No.	**MQC (2.00 µg/ml) Area of GBP**	
	Unextracted	**Extracted**
1	11855128	8942349
2	12044387	9307512
3	12039162	8913235
4	12160617	8900607
5	12026886	8741100
6	12092220	8811499
n	6	6
Mean	12036400.00	8936050.33
SD	101555.12	196631.07
%CV	0.84	2.20
%Recovery	74.24	
Sr. No.	**HQC (4.50 µg/ml) Area of GBP**	
	Unextracted	**Extracted**
1	25943141	18948482
2	26361578	19188069
3	26357843	19070589
4	25786432	20011910
5	25622017	19249242
6	25534676	19421973
n	6	6
Mean	25934281.17	19315044.17
SD	358083.31	377262.38
%CV	1.38	1.95
%Recovery	74.48	

TABLE NO: 4.18
VARIABILITY ACROSS QC LEVELS OF GBP

QC LEVELS	%Recovery
LQC	75.16
MQC	74.24
HQC	74.48
Mean	74.63
SD	0.476
%CV	0.64

TABLE NO: 4.19
RECOVERY OF INTERNAL STANDARD (LEV) (IS AMOUNT 1.50 µg/ml)

Sr. No.	Area of LEV	
	Unextracted	Extracted
1	5878075	6484337
2	5994692	6238048
3	6091646	6329058
4	6089236	6365513
5	5936550	6084339
6	6064166	6196985
7	5728743	6125954
8	5827817	6358164
9	5797186	6069732
10	5811422	6130189
11	5929188	6246940
12	5758359	6169999
13	5770043	6046114
14	5955326	6043754
15	5935133	6111543
16	5850428	6231866
17	5829306	5795219
18	5970797	5960257
n	18	18
Mean	5901006	6166001
SD	113183	163172
%CV	1.92	2.65
%Recovery	104.5	

4.3.8 Dilution integrity:

- Dilution integrity experiment was carried out at six replicate of two times diluted 2xULOQ (½ dilution) and four times diluted 2xULOQ (¼ dilution) samples. The samples were prepared and its concentrations were calculated against the previously bulk spiked calibration standards. The % CV for ½ dilutions and ¼ dilutions samples for GBP were 3.76 and 2.88, respectively (Table No. 4.20). The mean % nominal concentration for ½ dilutions and ¼ dilutions samples for GBP were 110.1 and 112.8, respectively (Table No. 4.20)

TABLE NO: 4.20

DILUTION INTEGRITY OF GBP

Conc. (µg/ml)	Dilution	Area Ratio	Calculated conc. (µg/ml)	% Nominal Conc.	Mean	SD	%CV
12.000	1/4(2ULOQ)	2.314	13.22	110.1	112.8	3.25	2.88
12.000	1/4(2ULOQ)	2.366	13.52	112.7			
12.000	1/4(2ULOQ)	2.345	13.40	111.6			
12.000	1/4(2ULOQ)	2.471	14.12	117.6			
12.000	1/4(2ULOQ)	2.294	13.10	109.2			
12.000	1/4(2ULOQ)	2.429	13.87	115.6			

Table No. 4.20 continue

12.000	1/2(2ULOQ)	4.788	13.68	114.0	110.1	4.14	3.76
12.000	1/2(2ULOQ)	4.753	13.58	113.2			
12.000	1/2(2ULOQ)	4.790	13.69	114.1			
12.000	1/2(2ULOQ)	4.398	12.57	104.7			
12.000	1/2(2ULOQ)	4.478	12.80	106.6			
12.000	1/2(2ULOQ)	4.539	12.97	108.1			

Underlined Values were outside the acceptance criteria of 85 - 115 % of their Nominal concentration.

4.3.9 Matrix effect:

- In order to ensure the effect of matrix through out the application of the method, plasma blanks obtained from six different lots (04 normal Heparinised, 01 lipemic and 01 Haemolysed) were spiked with GBP and LEV at LQC and HQC level. Three quality control samples at each level along with the set of calibration standards were analyzed and the % nominal concentration of the samples analyzed was represented in Table No. 4.21 for GBP.

TABLE NO: 4.21
MATRIX EFFECT FOR GBP

Sample ID	Area		Area Ratio	Calculated Conc. (µg/ml)	% Nominal Conc.
	GBP	LEV			
LQC (B225-08)	650607	5282209	0.123	0.158	105.3
LQC (B225-08)	545422	5387477	0.101	0.129	86.00
LQC (B225-08)	640750	5168602	0.124	0.159	106.0
LQC (B233-08)	610622	5748937	0.106	0.136	90.67
LQC (B233-08)	676296	5442613	0.124	0.159	106.0
LQC (B233-08)	597559	5371499	0.111	0.142	94.67
LQC (B235-08)	640966	5194270	0.123	0.158	105.3
LQC (B235-08)	626810	5188691	0.121	0.155	103.3
LQC (B235-08)	649784	5296223	0.123	0.157	104.7
LQC (B257-08)	635371	5269874	0.121	0.154	102.7
LQC (B257-08)	605975	5167406	0.117	0.150	100.0
LQC (B257-08)	619823	5085894	0.122	0.156	104.0
LQC (C001-08)	613001	5082831	0.121	0.154	102.7
LQC (C001-08)	615736	5268006	0.117	0.149	99.32
LQC (C001-08)	626823	5246560	0.119	0.153	102.0
LQC (D001-08)	600304	5033638	0.119	0.153	102.0
LQC (D001-08)	599510	5132926	0.117	0.149	99.33
LQC (D001-08)	620307	5208218	0.119	0.152	101.3
HQC (B225-08)	17133637	5141557	3.332	4.333	96.29
HQC (B225-08)	17359078	5108035	3.398	4.419	98.20
HQC (B225-08)	17579091	5219371	3.368	4.380	97.33
HQC (B233-08)	17464700	5104934	3.421	4.449	98.87
HQC (B233-08)	17903117	5278421	3.392	4.410	98.00
HQC (B233-08)	17661794	5241003	3.370	4.382	97.38
HQC (B235-08)	10326750	5233174	1.973	2.565	57.00
HQC (B235-08)	17417496	5151214	3.381	4.397	97.71
HQC (B235-08)	18019725	5319023	3.388	4.405	97.89

Table No. 4.21 continue

HQC (B257-08)	17289568	5028727	3.438	4.471	99.36
HQC (B257-08)	18163954	5325605	3.411	4.435	98.56
HQC (B257-08)	17342054	5405325	3.208	4.172	92.71
HQC (C001-08)	13654431	5373683	2.541	3.303	73.40
HQC (C001-08)	18340751	5109316	3.590	4.668	103.7
HQC (C001-08)	18048440	5248333	3.439	4.472	99.38
HQC (D001-08)	17843374	5101723	3.498	4.548	101.1
HQC (D001-08)	14638922	5142617	2.847	3.701	82.24
HQC (D001-08)	17966219	5142285	3.494	4.543	100.9

Underlined Values were outside the acceptance criteria of 85 – 115 % of their Nominal concentration.

4.3.10 Stability:

4.3.10.1 Stock solution stability.

- Stock solution stability was determined by comparing the peak areas of freshly prepared samples (comparison samples) with stability samples.

Stock solution stability of GBP (93 hrs at room temp. for main stock):

- GBP main stock solution was found to be stable at room temperature for 93 hrs with % mean change of 4.10 (Table No.4.22).

Stock solution stability of LEV (93 hrs at room temp. for main stock):

- LEV (IS) main stock solution was found to be stable at room temperature for 93 hrs with % mean change of -1.29 (Table No. 4.23).

Stock solution stability of GBP (93 hrs at 2-8˚C for main stock):

- GBP main stock solution was found to be stable at 2-8˚C for 93 hrs with % mean change of 5.38 (Table No.4.24).

Stock solution stability of LEV (93 hrs at 2-8˚C for main stock):

- LEV Main stock solution was found to be stable at 2-8˚C for 93 hrs with % mean change of -2.35 (Table No.4.25).

TABLE NO: 4.22
MAIN STOCK SOLUTION STABILITY OF GBP (93 hrs at room temp.)

Sr. No.	GBP(12.00 µg/ml)	
	Comp. sample area	Stability sample area
1	37965530	39658803
2	37885805	39813714
3	38242777	39070867
4	38073735	39884983
5	37874277	39330670
6	38241913	39890216
n	6	6
Mean	38047340	39608209
SD	167026	336657
% CV	0.44	0.85
% Mean Change	4.10	

TABLE NO: 4.23
MAIN STOCK SOLUTION STABILITY OF LEV (93 hrs at room temp.)

Sr. No.	LEV(1.50 µg/ml)	
	Comp. sample area	Stability sample area
1	6065366	6036516
2	6062324	6008907
3	6155877	5863199
4	5971748	5993892
5	5962427	5891232
6	6044741	6001840
n	6	6
Mean	6043747	5965931
SD	70970	70759
% CV	1.17	1.19
% Mean Change	-1.29	

TABLE NO: 4.24
MAIN STOCK SOLUTION STABILITY OF GBP (93 hrs at 2-8˚C)

Sr. No.	GBP(12.00 µg/ml)	
	Comp. sample area	Stability sample area
1	37965530	39114837
2	37885805	40092928
3	38242777	40095247
4	38073735	40644570
5	37874277	40215347
6	38241913	40394861
n	6	6
Mean	38047340	40092965
SD	167026	522901
% CV	0.44	1.30
% Mean Change	5.38	

TABLE NO: 4.25
MAIN STOCK SOLUTION STABILITY OF LEV (93 hrs at 2-8˚C)

Sr. No.	LEV(1.50 µg/ml)	
	Comp. sample area	Stability sample area
1	6065366	5829432
2	6062324	5914353
3	6155877	5875641
4	5971748	5931620
5	5962427	5958053
6	6044741	5901846
n	6	6
Mean	6043747	5901824
SD	70970	45017
% CV	1.17	0.76
% Mean Change	-2.35	

4.3.10.2 Bench top stability of GBP (at room temp. for 20 hrs):

- LQC and HQC samples were spiked in human plasma and were kept at room temperature for 20 hrs and were processed and analyzed along with previously bulk

spiked calibration standards, LQC and HQC samples. Concentrations were calculated to determine % mean change during stability period.

- GBP was found to be stable in LQC and HQC samples for 20 Hrs at room temperature with % mean change of 4.17 and 3.71, respectively (Table No. 4.26).

TABLE NO: 4.26
BENCH TOP STABILITY OF GBP (20 hrs at room temp.)

Sr. No.	LQC (0.15 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	0.147	98.00	0.160	106.7
2	0.149	99.33	0.151	100.7
3	0.149	99.33	0.156	104.0
4	0.145	96.67	0.156	104.0
5	0.151	100.7	0.157	104.7
6	0.146	97.33	0.144	96.00
n	6		6	
Mean	0.148		0.154	
% Mean Change	4.17			
Sr. No.	HQC (4.50 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	4.477	99.49	4.550	101.1
2	4.535	100.8	4.639	103.1
3	4.458	99.07	4.724	104.9
4	4.587	101.9	4.896	108.8
5	4.745	105.4	4.935	109.7
6	4.655	103.4	4.733	105.2
n	6		6	
Mean	4.576		4.746	
% Mean Change	3.71			

4.3.10.3 Process stability of GBP (at 5°C in auto sampler for 76 hrs):

- LQC and HQC samples were prepared and processed. These processed samples were kept in auto sampler for 76 hrs at 5°C, these samples were analyzed along with previously bulk spiked calibration standards, LQC and HQC samples concentrations were calculated to determine % mean change during stability period.
- GBP was found to be stable in LQC and HQC samples for 76 hrs at 5°C in auto sampler with % mean change of 0.77 and 5.44, respectively (Table No. 4.27).

TABLE NO: 4.27
PROCESS STABILITY OF GBP (at 5°C in auto sampler for 76 hrs)

Sr. No.	LQC (0.15 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	0.147	98.00	0.149	99.33
2	0.150	100.0	0.153	102.0
3	0.153	102.0	0.154	102.7
4	0.153	102.0	0.157	104.7
5	0.153	102.0	0.152	101.3
6	0.152	101.3	0.150	100.0
n	6		6	
Mean	0.151		0.153	
% Mean Change	0.77			
Sr. No.	**HQC (4.50 µg/ml)**			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	4.407	97.93	4.625	102.8
2	4.418	98.18	4.602	102.3
3	4.275	95.00	4.720	104.9
4	4.346	96.58	4.769	105.9
5	4.513	100.3	4.546	101.2
6	4.415	98.11	4.548	101.1
n	6		6	
Mean	4.396		4.635	
% Mean Change	5.44			

4.3.10.4 Freeze and thaw stability of GBP (after 4th cycle at -20°C):

- Samples were prepared at LQC and HQC levels, aliquoted and frozen at -20°C. Six samples from each concentration were subjected to three freeze and thaw cycles (stability samples). These samples were processed after 4th cycle and analyzed along with previously bulk spiked calibration standards, LQC and HQC samples (comparison samples). Concentrations were calculated to determine % mean change after 4th cycle.
- GBP was found to be stable in LQC and HQC samples after 4th cycle at -20°C with % mean change of 0.00 and -14.36, respectively (Table No. 4.28).

TABLE NO: 4.28
FREEZE AND THAW STABILITY OF GBP (after 4th cycle at -20°C)

Sr. No.	LQC (0.15 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	0.146	97.33	0.158	105.3
2	0.151	100.7	0.150	100.0
3	0.155	103.3	0.150	100.0
4	0.154	102.7	0.152	101.3
5	0.149	99.33	0.148	98.67
6	0.156	104.0	0.151	100.7
n	6		6	
Mean	0.152		0.152	
% Mean Change	0.00			

Table No. 4.28 continue

Sr. No.	HQC (4.50 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	4.536	100.8	2.463	54.73
2	4.583	101.8	4.132	91.82
3	4.610	102.4	4.197	93.27
4	4.567	101.5	4.361	96.91
5	4.627	102.8	4.283	95.18
6	4.476	99.47	4.029	89.53
n	6		6	
Mean	4.567		3.911	
% Mean Change	-14.36			

Underlined Values were outside the acceptance criteria of 85 – 115 % of their Nominal concentration.

4.3.10.5 Freeze and thaw stability of GBP (after 4th cycle at -70°C):

- Samples were prepared at LQC and HQC levels, aliquoted and frozen at -70°C. Six samples from each concentration were subjected to three freeze and thaw cycles (stability samples). These samples were processed after 4th cycle and analyzed along with previously bulk spiked calibration standards, LQC and HQC samples (comparison samples). Concentrations were calculated to determine % mean change after 4th cycle. GBP was found to be stable in LQC and HQC samples after 4th cycle at -70°C with % mean change of -5.71 and -6.07, respectively (Table No.4.29).

TABLE NO: 4.29
FREEZE AND THAW STABILITY OF GBP (after 4th cycle at -70°C)

Sr. No.	LQC (0.15 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	0.146	103.3	0.155	103.3
2	0.151	90.00	0.135	90.00
3	0.155	100.0	0.150	100.0
4	0.154	88.00	0.132	88.00
5	0.149	101.3	0.152	101.3
6	0.156	90.00	0.135	90.00
n	6		6	
Mean	0.152		0.143	
% Mean Change	-5.71			
Sr. No.	HQC (4.50 µg/ml)			
	Comparison samples		Stability samples	
	Sample conc.	% Nominal Conc.	Sample conc.	% Nominal Conc.
1	4.536	100.8	4.259	94.64
2	4.583	101.8	4.251	94.47
3	4.610	102.4	4.259	94.64
4	4.567	101.5	4.396	97.69
5	4.627	102.8	4.321	96.02
6	4.476	99.47	4.249	94.42
n	6		6	
Mean	4.567		4.289	
% Mean Change	-6.07			

4.3.11 Carry over check for GBP:

- No area response was observed or area response less than 20% and 5% of mean response of an extracted LLOQ standard was observed at the RT of analyte and internal standard (IS), respectively, in respective blank matrix and or blank solution (Table No. 4.30).

TABLE NO: 4.30
CARRY OVER CHECK FOR GBP

Sr. No.	Sample Name	Area		Area Ratio	Calculated Conc. (µg/ml)
		GBP	LEV		
1	EXTRACTED LLOQ	246416	6634503	0.037	0.051
2	EXTRACTED LLOQ	244374	6736462	0.036	0.049
3	BLANK PLASMA	NF	NF	NA	NA
4	EXTRACTED ULOQ	27080421	6731495	4.023	6.036
5	BLANK PLASMA	NF	NF	NA	NA
6	EXTRACTED ULOQ	27496928	6883295	3.995	5.994
7	BLANK PLASMA	NF	NF	NA	NA

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CHAPTER: 5 DEVELOPMENT AND VALIDATION OF EXTRACTIVE SPECTROPHOTOMETRIC METHODS FOR ESTIMATION OF GABAPENTIN IN PHARMACEUTICAL DOSAGE FORMS

5.1 INTRODUCTION TO EXTRACTIVE SPECTROSCOPY[1]:

- The addition of an amine in its ionized form to an ionized acidic dye, e.g. methyl orange or bromocresol purple, yields a salt (ion-pair) that may be extracted into an organic solvent such as chloroform or dichloromethane. The indicator dye is added in excess and the pH of the aqueous solution is adjusted if necessary to a value where both the amine and dye are in the ionized forms. The ion pair is separated from the excess indicator by extraction into the organic solvent, and the absorbance is measured at the λmax of the indicator in the solvent.
- Usually, the most intensely absorbing form of the indicator is measured, with the addition of acidified or basified ethanol. Alternatively, the absorbance of the indicator may be measured in aqueous solution after back extraction from the organic solvent. Because of higher molar absorptivity (ion-pairs) obtained by acid-dye method, this method is very sensitive.
- The correct choice of pH may permit the selective assay of the mixture of an amine and a quaternary ammonium salt.

5.2 EXPERIMENTAL WORK:

5.2.1 APPARATUS:

- A double beam UV-Visible Spectrophotometer (Shimadzu model UV-1700, Japan), attached to a computer software UV probe 2.10, with a spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells.
- Analytical balance (CP224S, Sartorius, Gottingen, Germany).
- Ultrasonic cleaner (Frontline FS 4, Mumbai, India).
- Corning volumetric flasks, pipettes of borosilicate glass were used in the study.

5.2.2 REAGENTS AND MATERIALS:

- GBP bulk powder was kindly gifted by Torrent research centre, Gujarat (India).
- The commercial fixed dose products containing 300 mg GBP were procured from the local pharmacy.
- AR grade chloroform (S.D. Fine Chemical Ltd., Mumbai, India).

- AR grade Bromocresol Green reagent and Bromothymol Blue reagent ((Finar Chemicals Ltd., India)
- Whatman filter paper no. 41 (Millipore, USA)

5.2.3 PREPARATION OF REAGENTS:

a) Preparation of diluent:

- Distilled water was used as diluent.

b) Preparation of buffer solutions:

- Phosphate buffer solution (pH 2.0):
- Potassium dihydrogen phosphate (13.6 mg) was accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 2.0 with HCl.
- Phosphate buffer solution (pH 2.5):
- Potassium dihydrogen phosphate (10 g) was accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 2.5 with HCl.
- Phosphate buffer solution (pH 3.0):
- Potassium dihydrogen phosphate (13.6 mg) was accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 3.0 with HCl.
- Phosphate buffer solution (pH 3.5):
- Potassium dihydrogen phosphate (13.6 mg) was accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 3.5 with HCl.
- Phosphate buffer solution (pH 4.0):
- Disodium hydrogen phosphate (504 mg) and Potassium dihydrogen phosphate (301 mg) were accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 4.0 with glacial acetic acid.
- Phosphate buffer solution (pH 4.5):
- Disodium hydrogen phosphate (504 mg) and Potassium dihydrogen phosphate (301 mg) were accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 4.6 with glacial acetic acid.
- Phosphate buffer solution (pH 5.0):
- Potassium dihydrogen phosphate (4 g) was accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 5.0 with 10 M KOH.
- Phosphate buffer solution (pH 5.5):
- Potassium dihydrogen phosphate (4 g) was accurately weighed and dissolved in water (100 ml). pH of solution was adjusted to 5.5 with 10 M KOH.

c) Preparation of bromocresol green dye (0.05% w/v):

- Bromocresol Green reagent (50 mg) was accurately weighed and 0.1 N NaOH (0.72 ml) was added. Ethanol (20 ml) was added and shaken well and then volume was made up to 100 ml with water.

d) Preparation of bromothymol blue dye (0.05% w/v):

- Bromothymol Blue reagent (50 mg) was accurately weighed and 0.02 M NaOH (4 ml) was added. Ethanol (95 %) (20 ml) was added and shaken well and then volume was made up to 100 ml with water.

e) Preparation of standard stock solutions:

- GBP (100 mg) was accurately weighed and transferred into 100 ml volumetric flask. Distilled water (100 ml) was added to get GBP standard stock solution (1 mg/ml). Working standard solution (200 µg/ml) was prepared by diluting GBP standard stock solution (20 ml) to 100 ml with diluent.

5.2.4 DEVELOPMENT OF THE METHOD:

- In colorimetric method for optimization, different dyes like, bromocresol green, bromothymol blue, methyl orange, thymol blue and phenolphthalein were prepared and used. But among them only BCG and BTB gave color with drug, hence BCG (Method I) and BTB (Method II) were used for development of the methods.

a) Optimization of conditions:

- Conditions under which reaction of GBP with dyes fulfill the essential requirements were investigated. All conditions studied were optimized at room temperature (25 ± 2^0C).

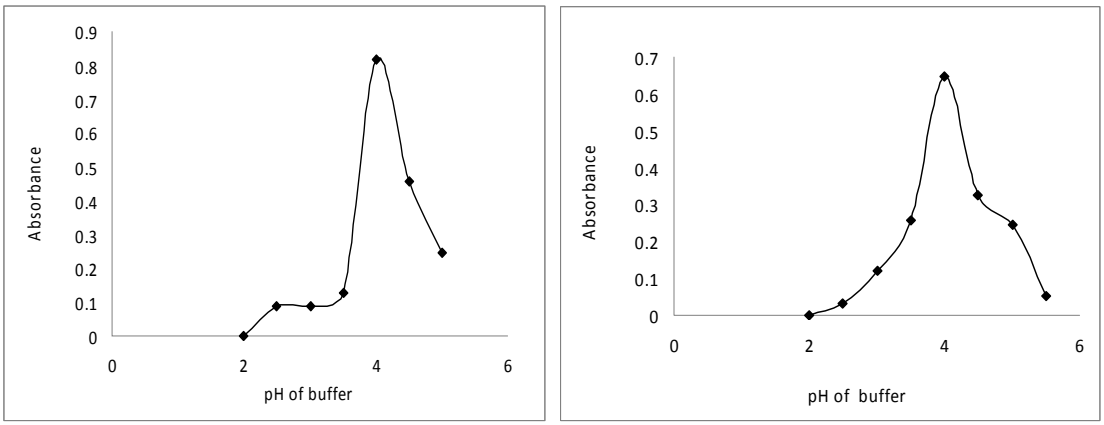
b) Selection of suitable pH buffer solution:

- Buffer solutions of different pH (2.0 to 5.5) were prepared. Working standard solution (5 ml) was pipette out and added to separating funnels. BCG solution (in excess) and buffer solutions (2 ml) of different pH were added to each. Shaken well and extracted with 10 ml of chloroform. Later the extracts were taken into 10 ml volumetric flasks, treated with anhydrous sodium sulphate and volume was made up with chloroform, and then absorbances were measured at 416 nm. Same procedure was applied for the BTB and then absorbances were measured at 421 nm. It was found that drug with BCG and BTB gave maximum absorbance at pH 4.0 (Figure 5.1 A and B)

c) Optimization of volume of buffer:

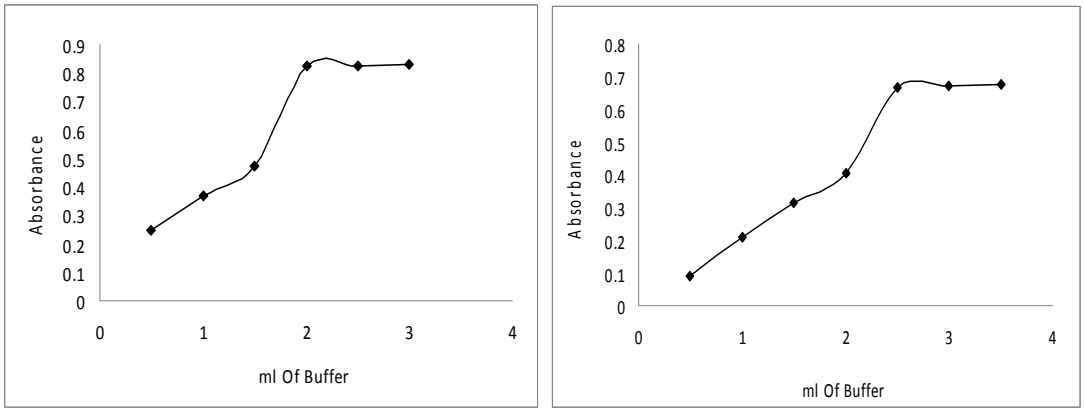
- Volume of buffer was optimized by changing volume of buffer and other parameters were kept constant. Buffer solution of pH 4.0 was used for optimization. Working standard solution (5 ml) was transferred in seven separating funnels, different volume of

buffer solution was added in the different separating funnels. BCG solution or BTB solution was added in excess each. Shaken well and extracted with 10 ml of chloroform. Later the extracts were taken into 10 ml volumetric flasks, treated with anhydrous sodium sulphate and volume was made up with chloroform, and then absorbances were measured at 416 nm and 421 nm for BCG and BTB, respectively. It was found that after addition of 2 ml of buffer in BCG and 2.5 ml of buffer in BTB, absorbance became constant. Hence 2 ml and 2.5 ml of buffer solution were optimized for BCG method and BTB method, respectively (Figure 5.2 A and B).



(A) **(B)**

Figure 5.1 Optimization of pH of Buffer for (A) GBP-BCG (B) GBP-BTB complex



(A) **(B)**

Figure 5.2 Optimization of volume of Buffer for (A) GBP-BCG (B) GBP-BTB complex

d) Stability study of drug dye complexes:

- The stability of the drug dye complexes was determined individually for both the dyes (BCG and BTB). Working standard solution (5 ml) was pipette out and added to a

separating funnels. Buffer solution (2 ml) and BCG solution (in excess) were added to each. Shaken well and extracted with 10 ml of chloroform. Later the extracts were taken into 10 ml volumetric flasks, treated with anhydrous sodium sulphate and made up the volume with chloroform. The absorbances were measured periodically at an interval of 30, 60, 90, 120, 180, 240, 300 and 360 minutes at 416 nm. Same procedure was applied for the BTB method using buffer solution (2.5 ml) and BTB solution (in excess), and then absorbances were measured at 421 nm. Finally it was found that BCG-GBP complex was stable at least for 6 hrs, whereas BTB-GBP complex was stable at least for 4 hrs.

5.2.5 METHOD VALIDATION:

a) Linearity:

Method I (BCG Method)

- From the working standard solution, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml were transferred to a series of separating funnels and buffer solution (2 ml) was added to each separating funnels, then BCG solution was added in excess and shaken well, and then 10 ml of chloroform was added to each and shaken well and kept for few minutes. Later the extracts were taken into 10 ml volumetric flasks, treated with anhydrous sodium sulphate and made up the volume with chloroform, and then absorbance of the solution was measured at 416 nm (Figure 5.3) against reagent blank. Final concentrations of analyzed solutions were 10 µg/ml to 120 µg/ml. The standard calibration plot was prepared to calculate the amount of the analyte drug in unknown samples.

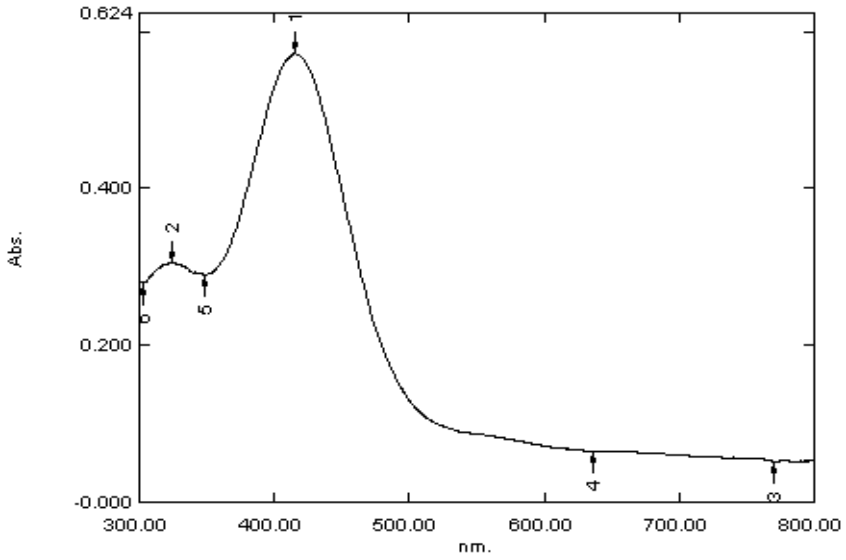


Figure 5.3 Representative absorption spectra of GBP-BCG showing λmax at 416 nm

Method II (BTB Method)

- From the working standard solution, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 ml were transferred to a series of separating funnels and buffer solution (2.5 ml) was added to each separating funnels, then BTB solution was added in excess and shaken well, and then 10 ml of chloroform was added to each and shaken well and kept for few minutes. Later the extracts were taken into 10 ml volumetric flasks, treated with anhydrous sodium sulphate and made up the volume with chloroform, and then absorbance of the solution was measured at 421 nm (Figure 5.4) against reagent blank. Final concentrations of analyzed solutions were 40 µg/ml to 90 µg/ml. The standard calibration plot was prepared to calculate the amount of the analyte drug in unknown samples.

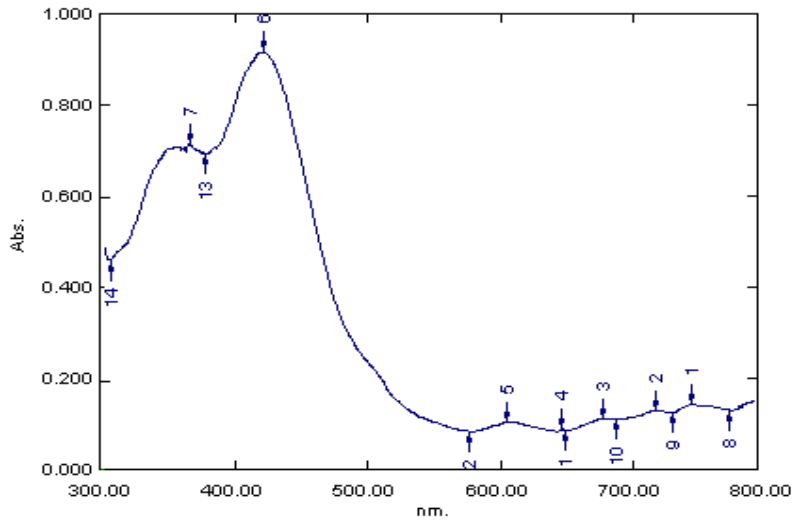


Figure 5.4 Representative absorption spectra of GBP-BTB showing λmax at 421 nm

b) Accuracy (% Recovery):

- The accuracy of the proposed methods was performed by calculating recovery of GBP by the standard addition method. Known amounts of standard solutions of GBP were added at 50, 75 and 100% levels to prequantified sample solutions of 40 µg/ml GBP for BCG and BTB both. At each level of the amount 3 determinations were performed. The amount of GBP was estimated by applying obtained values to regression equation.

c) Method precision (Repeatability):

- The precision of the instrument was checked by repeated scanning and measurement of the absorbance of solutions of 50 µg/ml GBP (n = 6) and 70 µg/ml GBP (n = 6) for BCG and BTB, respectively without changing the parameters for the method. The repeatability was expressed in terms of % relative standard deviation (%RSD).

d) Intermediate precision (Reproducibility):

- The intraday and interday precision of the proposed methods were performed by analyzing the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 6 different concentrations of standard solutions of GBP (20, 40, 60, 80, 100 and 120 µg/ml) for BCG and GBP (40, 50, 60, 70, 80 and 90 µg/ml) for BTB. The results were reported in terms of %relative standard deviation (%RSD).

e) Limit of detection and Limit of quantification:

- The limit of detection (LOD) and limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise (i.e. 3.3 for LOD and 10 for LOQ) ratio using the following equations designated by International Conference on Harmonization (ICH) guideline:

$$LOD = 3.3 \times \sigma/S \text{ and } LOQ = 10 \times \sigma/S$$

Where, σ = the standard deviation of the response, S = slope of the calibration curve

5.3 RESULTS AND DISCUSSION:

METHOD VALIDATION:

5.3.1 Linearity:

- Linear correlation was obtained between absorbance versus concentrations of GBP in the ranges of 10-120 µg/ml for BCG and 40-90 µg/ml for BTB. The linearity of the calibration curve was validated by the high values of correlation coefficient of regression line (Table No. 5.1; Figure 5.5 and 5.6).

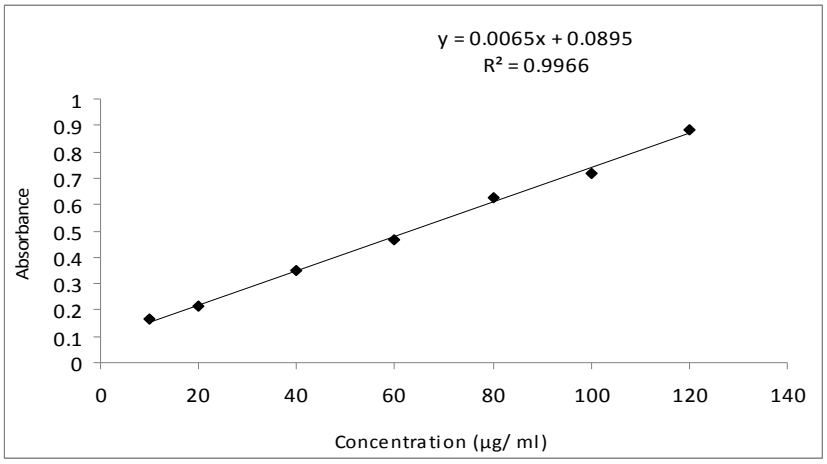


Figure 5.5 Calibration curve for GBP-BCG

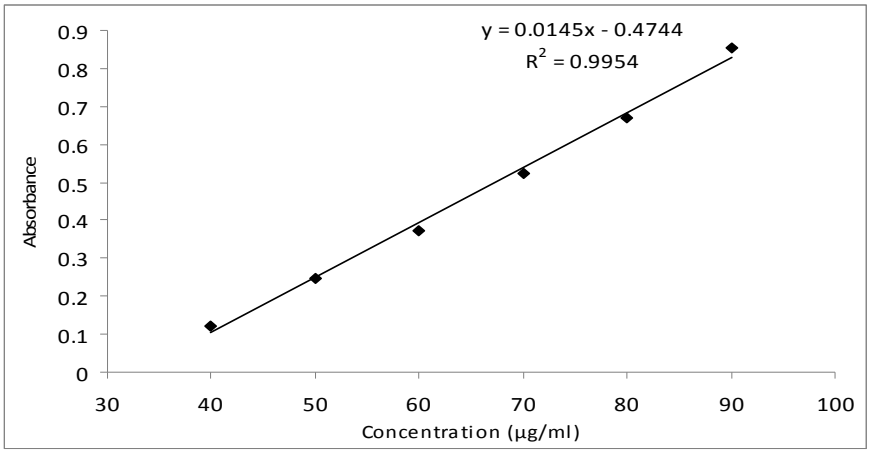


Figure 5.6 Calibration curve for GBP-BTB

TABLE NO: 5.1
REGRESSION ANALYSIS DATA AND SUMMARY OF VALIDATION PARAMETERS FOR THE PROPOSED METHODS

Parameters	BCG	BTB
λ_{max}, nm	416	421
Beer Lambert's law limits, µg/ml	10-120	40-90
Molar absorptivity*	1627.70	1123.72
Sandell's sensitivity, µg/cm^2 /0.001 Absorbance unit*	0.1138	0.1750
Regression equation y=mx+c	y = 0.0065x + 0.0895	y = 0.0145x - 0.4744
Slope (m)	0.0065	0.0145
Intercept (c)	0.0895	-0.4744
Correlation coefficient (r^2)	0.9966	0.9954
Limit of detection (LOD), µg/ml	2.90	10.86
Limit of quantification (LOQ), µg/ml	8.77	32.91
Repeatability (%RSD, n=6)	0.357	0.330
Precision (%RSD)		
Interday (n = 3)	0.77-1.90	0.87-1.77
Intraday (n = 3)	0.14-1.08	0.31-1.63

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* Mean of six determinations

5.3.2 Accuracy (% Recovery):

- The recovery experiments were performed by the standard addition method. The mean recovery of GBP was 99.23 $\pm$ 0.77 to 100.4 $\pm$ 0.77 and 99.23$\pm$ 0.77 to 99.91 $\pm$ 1.07 for BCG in capsule and tablet dosage form, respectively (Table No. 5.2 and 5.3). The mean recovery of GBP was 99.62 $\pm$ 0.29 to 101.6 $\pm$ 0.18 and 98.78 $\pm$ 0.11 to 101.4 $\pm$ 0.05 for BTB in capsule and tablet dosage form, respectively (Table No. 5.2 and 5.3).The low

value of standard deviation indicates that the proposed methods are accurate. Results of recovery studies are shown in Table No. 5.2 and 5.3.

TABLE NO: 5.2
DRUG RECOVERY STUDY IN CAPSULE DOSAGE FORM

Drug-Dye Complex	Level	Amount of sample taken (µg/ml)	Amount of standard spiked (%)	Mean % Recovery ± SD* (n=3)
GBP-BCG	I	40	50 %	99.23 ± 0.77
	II	40	75 %	100.4 ± 0.77
	III	40	100 %	100.3 ± 1.60
GBP-BTB	I	40	50 %	99.62 ± 0.29
	II	40	75 %	99.88 ± 0.25
	III	40	100 %	101.6 ± 0.18

TABLE NO: 5.3
DRUG RECOVERY STUDY IN TABLET DOSAGE FORM

Drug-Dye Complex	Level	Amount of sample taken (µg/ml)	Amount of standard spiked (%)	Mean % Recovery ± SD* (n=3)
GBP-BCG	I	40	50 %	99.23 ± 0.77
	II	40	75 %	99.36 ± 1.18
	III	40	100 %	99.91 ± 1.07
GBP-BTB	I	40	50 %	98.78 ± 0.11
	II	40	75 %	99.12 ± 0.25
	III	40	100 %	101.4 ± 0.05

5.3.3 Method precision (Repeatability):

- The RSD value of method precision for GBP was found to be 0.357 % for BCG and 0.330 % for BTB. (Table No. 5.4). Low relative standard deviation was indicates that the proposed methods are repeatable.

TABLE NO: 5.4
PRECISION DATA FOR GBP

Sr. No.	Absorbance of GBP	
	BCG (GBP: 50 µg/ml)	BTB (GBP: 70 µg/ml)
1	0.412	0.521
2	0.412	0.52
3	0.414	0.522
4	0.413	0.524
5	0.411	0.521
6	0.415	0.519
Mean	0.413	0.521
SD	0.001	0.002
% RSD	0.357	0.330

5.3.4 Intermediate precision (Reproducibility):

- The low % RSD values of interday (0.77% -1.90 % for BCG and 0.87% -1.77% for BTB) and intraday (0.14%-1.08 % for BCG and 0.31%-1.63% for BTB) variations reveal that the proposed methods provide acceptable intraday and interday variation for GBP at different concentration levels (calibration range concentration for BCG and BTB) (Table No. 5.1).

5.3.5 Limit of detection (LOD) and Limit of quantification (LOQ):

- LOD and LOQ values for GBP were found to be 2.90 µg/ml and 8.77 µg/ml, respectively for BCG and 10.86 µg/ml and 32.91 µg/ml for BTB, respectively. These data show that this method is sensitive for the determination of GBP (Table No. 5.1).

5.3.6 Assay of the pharmaceutical formulations:

- The proposed validated methods were successfully applied to determine GBP in its dosage forms. The results obtained were comparable with the corresponding labeled amount (Table No. 5.5 and 5.6). No interference of the excipients with the absorbance of interest appeared; hence the proposed methods are applicable for the routine estimation of GBP in pharmaceutical dosage forms.

TABLE NO: 5.5
ANALYSIS OF MARKETED FORMULATION (CAPSULE DOSAGE FORM) OF GBP BY PROPOSED METHODS (n = 6)

Sample No.	Label Claim	Amount Found		% Label Claim	
	GBP (mg/cap)	GBP (mg/cap)		GBP (mg/cap)	
		BCG	BTB	BCG	BTB
1	300	301.1	298.6	100.4	99.52
2	300	304.8	297.7	101.6	99.22
3	300	302.0	297.0	100.7	99.01
4	300	301.1	297.9	100.4	99.32
5	300	297.3	296.7	99.11	98.91
6	300	304.8	298.8	101.6	99.62
Mean				100.6	99.27
SD				0.93	0.28

TABLE 5.6
ANALYSIS OF MARKETED FORMULATION (TABLET DOSAGE FORM) OF GBP BY PROPOSED METHODS (n = 6)

Sample No.	Label Claim	Amount Found		% Label Claim	
	GBP (mg/tab)	GBP (mg/tab)		GBP (mg/tab)	
		BCG	BTB	BCG	BTB
1	300	300.5	295.1	100.2	98.36
2	300	294.9	294.2	98.31	98.07
3	300	303.2	293.6	101.1	97.87
4	300	296.8	295.4	98.92	98.46
5	300	294.0	295.4	98.00	98.46
6	300	299.5	294.8	99.85	98.27
Mean				99.38	98.25
SD				1.18	0.24

5.4 REFERENCES:

1. Beckett AH, Stenlake JB. Practical pharmaceutical chemistry. 4th ed.; 2004: 2, p.304.

CHAPTER 6: SUMMARY AND CONCLUSION

6.1 SUMMARY:

- Methods including LC-MS/MS, GC-MS, HPLC, HPTLC, Capillary Electrophoresis, colorimetric methods were reviewed for the estimation of GBP in biological fluids and pharmaceutical formulations.
- LC-MS/MS method for estimation of GBP in human plasma was developed.
- The developed LC-MS/MS method was validated for linearity, accuracy, interday and intraday precision, matrix effect, dilution integrity, freeze and thaw stability, stock solution stability, recovery, bench top stability, process stability.
- Extractive spectrophotometric methods based on formation of ion pair between GBP and dyes were developed using BCG and BTB dye.
- Dye, pH of buffer, volume of buffer, volume of dye was optimized to get maximum absorbance of resulting solution.
- The developed extractive spectrophotometric methods were validated for linearity, limit of detection, limit of quantification, recovery (accuracy), reproducibility, interday and intraday precision.
- Finally the developed validated extractive spectrophotometric methods were applied in pharmaceutical dosage forms including capsule and tablets.

6.2 CONCLUSION:

6.2.1 LC-MS/MS METHOD:

- The results of the method validation for GBP indicated that the analytical method was valid for the analysis of GBP with a calibration range of 0.05 µg/ml to 6.00 µg/ml in human plasma using LEV as internal standard.
- From results of matrix effect, it was concluded that the method can be used for estimation of GBP in human plasma without any interferences of biological matrix.

6.2.2 EXTRACTIVE SPECTROPHOTOMETRIC METHODS:

- During the course of study, it was observed that acidic solution of the drug formed colored ion-association complexes with BCG and BTB which were soluble in chloroform. This property of the drug was followed for the development of extractive spectrophotometric methods for analysis of drug. The complex of GBP with BCG and

BTB showed λ_{max} at 416 nm and 421 nm, respectively. These developed methods were used for the estimation

of GBP from two formulations. Both methods involve formation of ion-associated complex with BCG and BTB at pH 4.0 exhibiting λ_{max} at 416 nm and 421 nm, respectively.

- The proposed methods were based on addition of an amine in its ionized form to an ionized dye, yield a salt (ion-pair) that was extracted into an organic solvent such as chloroform or dichloromethane. The indicator dye was added and the pH of the aqueous solution was adjusted to a value where both the amine and dye were in the ionized forms. The ion pair was extracted by extraction into the organic solvent. In these methods Beer's law was obeyed with BCG and BTB in the concentration range of 10-120 µg/ml and 40-90 µg/ml, respectively.
- The optimum conditions for color development had been established by varying the different parameters involved. For testing the accuracy and reproducibility of the proposed methods, recovery studies were performed. The data obtained by recovery studies indicate non-interference from the excipients used in the formulations. The percentage recoveries were close to 100%. This study revealed that the common excipients and other additives such as lactose, starch, gelatin, talc and magnesium stearate that are usually present in the tablet dosage forms do not interfere in the analysis. Thus, it can be concluded that the proposed methods are found to be simple, precise and accurate that can be used for the determination of GBP in their pharmaceutical dosage forms in a routine manner.

CHAPTER 7: PRESENTATIONS AND PUBLICATIONS

7.1 PAPER COMMUNICATED FOR PUBLICATION:

1. D.B.PATEL*, S.A.PATEL, R.V.SHAH, N.J.PATEL. Extractive Spectrophotometric Methods for the Determination of Gabapentin in Pharmaceutical Dosage Forms. Indian J Pharm Sci.